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(54) Title: CONTROL OF NITRIC OXIDE BIOACTIVITY BY PERFLUOROCARBONS

(57) Abstract: Perfluorocarbons are used to control nitric oxide metabolism, either to inhibit nitric oxide activity or to potentiate the effects of nitric oxide. Perfluorocarbons can be used, for example, to treat hypotension and vasoplegia in septic shock, to protect against myocardial ischemia-reperfusion injury, to treat hypertension, and to provide antiplatelet effects.

CONTROL OF NITRIC OXIDE BIOACTIVITY BY PERFLUOROCARBONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority from provisional application Serial No. 60/411,828, filed September 19, 2002. The entire contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

[0002] The present invention is directed to the use of perfluorocarbons to regulate bioactivity of nitric oxide in animals, either to inhibit the activity of nitric oxide or to enhance the activity of nitric oxide.

BACKGROUND OF THE INVENTION

[0003] Nitric oxide is synthesized enzymatically from L-arginine by almost all tissues of the body, including brain, peripheral nervous system, smooth muscle, kidney, vascular, lung, uterus, etc. Nitric oxide is involved in many vascular functions including vasorelaxation and blood clotting. (Moncada et al., 1993). Heme proteins such as guanylyl cyclase serve as primary targets for nitric oxide (Bredt et al., 1994).

[0004] There is a substantial body of evidence from animal and human studies that a deficiency in nitric oxide

contributes to the pathogenesis of a number of diseases, including hypertension, atherosclerosis, and diabetes.

[0005] Depending upon the rate, timing, and spatial distribution of nitric oxide production, as well as the chemical microenvironment (e.g., presence of ROS, redox status of the cell), nitric oxide acts either as a direct signaling messenger or as an indirect toxic effector via the formation of various reactive nitrogen species. Indeed, both functions may be occurring at the same time, i.e., spatially targeted production of nitric oxide may simultaneously enhance the antimicrobial function of the neutrophil respiratory burst while protecting host cells from oxidant injury and, perhaps, preventing the uncontrolled explosion of the inflammatory response (e.g., down-regulation of nuclear factor κ -B activation). At other times, the cytotoxic potential of reactive nitrogen species may turn against the host, as has been documented in some murine models of infection, such as Influenza A pneumonitis or intestinal and liver injury caused by *T. gondii*. (Feihl et al., 2001).

[0006] Nitric oxide was originally described as the principal endothelium-derived relaxing factor, but it is now known to subserve a variety of functions throughout the body, both physiological and pathophysiological. Among its diverse functions, nitric oxide has been implicated in

neurotransmission, immune regulation, vascular smooth muscle relaxation, and inhibition of platelet aggregation.

[0007] Nitric oxide has been found to activate soluble guanylate cyclase. Guanylate cyclase exists in two forms in the cell: a soluble isoform within the cytosol and a particulate isoform which is membrane-associated. Purified soluble guanylate cyclase can be activated *in vitro* by nitric oxide-donating compounds. Once the enzyme has been activated, there is accumulation of cGMP which, in vascular smooth muscle cells, is accompanied by relaxation and hence vasodilation.

[0008] In other cell types, the accumulation of cGMP is accompanied by different physiological effects. cGMP can be formed by soluble guanylate cyclase in platelets, following stimulation by nitric oxide derived either from the vascular endothelium or from endothelial-type NOS within the platelets themselves, leading to inhibition of platelet aggregation. In the central nervous system, nitric oxide can be formed in the postsynaptic nerve cell following activation of N-methyl-D-aspartate (NMDA) receptors by glutamate. This nitric oxide diffuses out and acts upon guanylate cyclase in one or more neighboring neurons, including the presynaptic nerve cells, thus acting as part of a feedback loop. In one model of brain activity, such a retrograde messenger is involved in the building up of long-term memory. In addition, nitric oxide in

the brain can act upon nerve cells other than the presynaptic nerve cell, causing a variety of effects: neuroprotection, long-term potentiation (an activity-dependent increase in synaptic strength), and long-term depression (a long-lasting depression of parallel fiber synapses following repeated excitation by climbing fibers of Purkinjie cells). It has also been suggested that overproduction of nitric oxide may cause neurotoxicity and certain degenerative conditions, such as Alzheimer-type dementia, either because of its radical nature or because it can generate peroxynitrite.

[0009] Nitric oxide is generated in the course of inflammatory and immune reaction, both by macrophages and by neutrophils. The role of these cells is quite extensive, and includes phagocytic and non-phagocytic destruction of foreign or damaged cells. These processes involve the production of large quantities of nitric oxide, which is cytotoxic.

[0010] It is possible that either an overabundance or a deficiency of nitric oxide is involved in many other pathological problems in females, such as preeclampsia, preterm labor, climacterium, pregnancy-induced diabetes, and postpartum hemorrhage. Nitric oxide overabundance or deficiency may also be associated with coronary artery disease, cancer, and behavioral and digestive problems.

[0011] Premenopausal women have a lower incidence of cardiovascular disease than men. After menopause, the incidence of cardiovascular disease increases progressively, as the risk of coronary heart disease rapidly increases after cessation of ovarian function. These changes are thought to be hormonally mediated and related to the decrease in production of both estrogen and progesterone. Since nitric oxide is very important in control of vascular function, a decrease in nitric oxide production or action is related to pathophysiological changes in blood vessels, *i.e.*, cardiovascular diseases associated with hypertension and atherosclerosis. The steroid hormones regulate nitric oxide synthesis. Thus, it is believed that nitric oxide may mediate all, or at least some, of the actions of the steroid hormones to prevent cardiovascular disease in premenopausal women. It may thus be possible to administer a nitric oxide donor to prevent cardiovascular disease as part of or instead of hormone replacement therapy, thus minimizing or eliminating any undesirable side effects of hormone replacement therapy.

[0012] Nitric oxide is also very much involved in the control of blood clotting. Nitric oxide and its donors are potent inhibitors of coagulation (*e.g.*, reviewed in Richardson G. and Benjamin N. (2002)).

[0013] In addition, nitric oxide has been implicated in bone remodeling. Bone remodeling disorders such as osteoporosis and osteoarthritis are frequently associated with perturbations in the interaction between local and systemic bone-remodeling regulatory pathways. Postmenopausal bone loss associated with diminished steroid hormones is correlated with increased levels of cytokines. In addition, both estrogen and progestins are effective in preventing postmenopausal bone loss (Garfield, et al., 1999).

[0014] Bone-degrading osteoclasts arise from cells within the monocyte macrophage lineage. Excessive osteoclast activity leads to high levels of bone destructions and osteoporosis. Although these cells have the unique ability to resorb bone, they share various characteristics with macrophages (Garfield, et al., 1999).

[0015] Macrophages release nitric oxide in response to inflammatory cytokines and agents. It has been suggested that osteoclasts, like macrophages, synthesize nitric oxide. In models of osteoporosis, nitric oxide inhibition potentiated the loss of bone mineral density by suppressing osteoclast activity and bone resorption. There have also been studies which demonstrated that nitric oxide is produced by chondrocytes. (Garfield, et al., 1999).

[0016] Since inhibition of osteoclastic activity is a major aim in treating and preventing osteoporosis, Paget bone disease and rheumatoid arthritis, nitric oxide donors may be useful in treating and preventing these conditions.

[0017] Presently, there are only three nitric oxide donor compounds that are used clinically: nitroglycerin, amyl nitrite, and sodium nitroprusside. Nitroglycerin is available in tablets or sprays for sublingual use, intravenously, or in patch form. Amyl nitrite is formulated as an inhalant and is usually used in breakable capsules. Sodium nitroprusside is limited to intravenous infusion. Nitric oxide donors are currently used for treating angina pectoris due to coronary artery disease (nitroglycerin or amyl nitrite) and control of blood pressure associated with myocardial infarction or surgical procedures (nitroglycerin or sodium nitroprusside).

[0018] Unfortunately, conventionally available nitric oxide donor compounds have a short duration of action, a short half-life, a lack of tissue specificity, development of tolerance, and accumulation of toxic substances (cyanide for sodium nitroprusside).

[0019] Nitric oxide is a gas with low solubility in water and aqueous solutions. Although nitric oxide is a free radical that is highly unstable in vivo, it does not interact

directly with most biological substrates and commonly used organic solvents.

[0020] An important physiologically significant component of nitric oxide biochemistry involves formation of thionitrite esters with free thiols (S-nitrosothiols: RS-NO). Low molecular weight S-nitrosothiols, e.g., S-nitrosoglutathione (GS-NO), S-NO-cysteine (S-NO-Cys) and nitroso derivatives of proteins such as albumin and hemoglobin (Hb) exert nitric oxide-like activity *in vivo*. They cause arterial and venous smooth muscle relaxation, inhibit platelet aggregation, and activate guanylate cyclase (Stamler et al., 1992; Ignarro et al., 1981; Keaney et al., 1993; Stamler, 1992; Scharfstein et al., 1994; Jia et al., 1996).

[0021] Vasoactive S-nitrosothiols are known to be generated *in vivo* (Stamler et al., 1992; Ignarro et al., 1981; Keaney et al., 1993; Stamler, 1992; Scharfstein et al., 1994; Jia et al., 1996; Clancy et al., 1994; Gaston et al., 1998). The originally reported amount of total S-nitrosothiols in human plasma was 7 μ M (Stamler et al., 1992). Many subsequent measurements (Rafikova et al., 2002) detected from 40 nM to 1 μ M of plasma S-nitrosothiols in humans and rodents under noninflammatory conditions (Hogg et al., 2002; Marzinzig, 1997; Tsikas et al., 1999). Since S-nitrosothiol compounds are relatively stable and can release nitric oxide when

required via reactions with transition metal ions or other reducing agents (Singh et al., 1996; Kashiba-Iwatsuki et al., 1997; Aleryani et al., 1998; Nikitovic et al., 1996), they are envisioned as a buffering system that controls intra- and extra-cellular activities of NO and magnify the range of its action. Once formed, S-nitrosothiols can directly transfer the nitrosyl cation (NO^+) to another thiol via the so-called transnitrosation reaction, which ensures the dynamic state of S-nitrosothiols *in vivo* (Ignarro et al., 1981; Jourd'heuil et al., 2000; Tsikas et al., 2001).

[0022] The properties of S-nitrosothiols (RSNOs) are similar to those of nitric oxide. They are involved in smooth muscle cell relaxation, platelet deactivation, immunosuppression, neurotransmission, and host defense. Although they have not yet been used therapeutically, there are data to suggest that S-nitrosothiols have a possible place in the management of a variety of diseases.

[0023] At present, the only commercially available S-nitrosothiols are S-nitroso-N-acetylpenicillamine (SNAP), N-acetyl-S-nitrosopenicillamyl-S-nitrosopenicillamine, and GSNA. None of these compounds has as yet been used therapeutically in animals or humans. One of the main problems with using S-nitrosothiols is their unpredictable rate of decomposition in physiological vehicles, which can

occur in the presence of copper and other divalent metals, by enzymatic degradation and as a result of transnitrosation.

[0024] Other S-nitrosothiols proposed as drugs are SNO-albumin, which acts as a reservoir of nitric oxide. It is formed when transnitrosation occurs between albumin and low-molecular-mass RSNOs, such as GSNO or SNO-cysteine. However, the cysteine residues in native albumin are few and are hidden inside the molecule. Different forms of poly-SNO-albumin have been prepared by covalent modification of albumin prior to nitrosylation.

[0025] Poly-SNO-albumin has been shown to inhibit human vascular smooth muscle cell proliferation in culture to a greater extent than both SNO-albumin and the NONOates.

[0026] Captopril, an angiotensin-converting enzyme (ACE) inhibitor, is a reduced thiol and can be nitrosated. It still acts as an ACE inhibitor in this form, but it also functions as a nitric oxide donor, being more effective than captopril itself in decreasing acute and chronic elevation of blood pressure in rats. SNO-captopril can also take part in transnitrosation reactions, transferring its nitroso moiety to haem proteins.

[0027] Tissue plasminogen activator (tPA) is another drug used in routine clinical practice that can undergo nitrosation. This has no effect on its catalytic ability,

fibrin stimulation, binding to fibrinogen, or interaction with plasminogen activator inhibitor-1. It does, however, have vasodilatory and anti-platelet effects by enhancing cGMP production. SNO-tPA was compared with tPA in cats in which ischemia/reperfusion injuries were induced. Treatment with SNO-tPA lowered the amount of myocardial necrosis and improved preservation of endothelial function, as assessed by relaxation in response to acetylcholine.

[0028] Von Willebrand factor is involved in platelet adhesion. A fragment of von Willebrand factor has been found to have a point mutation, where arginine is replaced by cysteine, and thus can be nitrosated. The fragment itself has anti-platelet properties, but nitrosation improves its ability to inhibit platelet aggregation and adhesion, both *in vitro* and in an *ex vivo* rabbit model. All of these effects were mediated both by enhancement of cGMP levels and by decreased binding to glycoprotein (Gp) Ib receptors.

[0029] Platelets play a significant role in both the development and the clinical presentation of vascular disease. Currently available agents that reduce platelet activation and adhesion, including aspirin, copidogrel, and abciximab, result in improved outcomes in patients with acute coronary syndromes. However, absolute levels of morbidity and mortality remain high. All RSNOs have been shown to influence

platelet function. cGMP is involved in the anti-platelet effects of RSNOs, but, for GSNO, at least, other mediators also appear to be involved. Unlike organic nitrates, which can also lower platelet activity, but at high doses, RSNOs can achieve this in healthy human volunteers in doses that do not affect vascular tone.

[0030] Platelet activation, as measured by levels of expression of the adhesion molecule P-selectin and the GpIIb/IIIa receptor, is increased in acute coronary syndromes. This phenomenon is seen even in the presence of aspirin. In a small clinical trial, it was found that GSNO significantly lowered levels of platelet activity. Glyceryl trinitrate also achieved this effect but, because it also induced hypotension, it was less well tolerated.

[0031] Interventional treatment of vascular disease using balloon angioplasty and stenting or coronary artery bypass grafting results in platelet activation. This is thought to play a role in the re-stenotic process observed following percutaneous intervention and in graft failure after surgery. SNO-albumin delivered locally to an area of balloon-injured rabbit femoral artery reduced platelet deposition and the subsequent development of neointimal hyperplasia. Local delivery of SNO-albumin using stent-based rather than catheter-based therapy was also found to reduce platelet

adhesion following deployment into pig carotid arteries. In a small clinical study, intracoronary infusion of GSNO prior to percutaneous transluminal coronary angioplasty prevented the increases in platelet P-selectin and GpIIb/IIIa expression usually seen within five minutes of the procedure, without altering blood pressure. Coronary artery bypass grafting is also associated with platelet activation and consumption, which can lead to post-operative bleeding. GSNO decreased the uptake of platelets by both arteries and veins *in vitro* and in patients undergoing coronary artery bypass grafting.

[0032] One of the main complications of carotid endarterectomy is cerebral infarction, often caused by platelet emboli. The procedure itself results in the removal of the endothelium, leaving a potent thrombogenic surface on which platelet adhesion and aggregation occurs. Asymptomatic microemboli can be detected by Doppler ultrasonography, and their frequency correlates with the risk of early stroke. In small studies, intravenous GSNO given either peri-operatively or post-operatively reduced the frequency of microemboli compared with placebo.

[0033] Like nitric oxide, RSNOs relax vascular smooth muscle cells. Another therapeutic use for RSNOs is in managing subarachnoid hemorrhage, a condition associated with cerebral vasospasm. This vascular response was significantly

reduced by an infusion of SNAP in a rat model of subarachnoid hemorrhage.

[0034] Coronary artery bypass grafting involves the use of both saphenous vein and internal mammary arteries as bypass conduits. Handling of these tissues can include vasospasm, potentially leading to early graft occlusion. Rings of conduit taken from patients undergoing coronary artery bypass grafting were exposed to GSNO and RIG200, another RSNO, and their vascular responses were compared with that seen after exposure to GTN. The RSNOs were found to significantly reduce the degree of vasospasm in these tissue sections compared with GTN.

[0035] Reperfusion of ischemic tissue leads to inflammatory responses and endothelial cell dysfunction. RSNOs have been shown to improve end-organ recovery in models of ischemia/reperfusion injury in the heart and the liver.

[0036] Thus, RSNOs have many potential roles in the treatment of vascular diseases, limiting the complications of platelet activation, of vasospasm, and of ischemia/reperfusion. These agents may be more effective than currently available nitric oxide donors, such as organic nitrates, which have generally shown little benefit apart from symptomatic relief.

[0037] Normal levels of RSNOs detected in bronchoalveolar lavage fluid are approximately 25 μ M. RSNOs relax bronchial smooth muscle, inhibiting the broncho-constrictor effects of methacholine on segments of human airway. As with their anti-platelet actions, cGMP appears to mediate only part of this bronchodilatory effect of RSNOs.

[0038] Airway RSNO levels are altered in disease states. In patients with pneumonia, the mean concentration was 0.4 μ M, higher than that seen in healthy controls. In patients with asthma, nitric oxide levels in exhaled breath are higher than normal, but airway RSNO levels are much reduced as compared with healthy controls.

[0039] Like nitric oxide, RSNOs appear to play a role in host defense, affecting both bacteria and viruses. Macrophages produce nitric oxide from L-arginine to exert a cytostatic effect in limiting the complications of platelet activation, of vasospasm, and of ischemia/reperfusion. These agents may be more effective than currently available nitric oxide donors, such as organic nitrates, which have generally shown little benefit apart from symptomatic relief.

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[0042] Like nitric oxide, RSNOs appear to play a role in host defense, affecting both bacteria and viruses. Macrophages produce nitric oxide from L-arginine to exert a cytostatic effect on *Trypanosoma musculi*. This effect is not seen in the absence of albumin, or in the presence of antibodies to SNO-cysteine, suggesting that the nitric oxide reacts with albumin to form SNO-albumin and then a trans-nitrosation reaction with cysteine occurs to form SNO-cysteine, the active moiety. RSNOs are toxic to a mutant form of *Salmonella typhimurium* in which intracellular homocysteine levels are depleted.

[0043] Both nitric oxide and SNAP inhibit HIV-1 protease, an enzyme involved in the replication of this virus. When nitric oxide reacts with the enzyme, it forms nitrosothiols with the two cysteine residues in the protein molecule. This

effect on HIV-1 is cGMP-independent and additive with that of zidovudine.

[0044] GSNO, SNAP, and SNO-captopril inactivate a protease enzyme in the human rhinovirus, which causes the common cold. This effect probably involves a transnitrosation reaction.

[0045] Accordingly, RSNOs can be used to treat conditions ranging from the common cold to AIDS.

[0046] RSNOs are inhibitors of gastrointestinal smooth muscle function. Their formation in the gut is thought to occur directly as a result of nitrosation of SH groups of low-molecular-mass thiols. Inhibition of motor activity in the duodenum and sphincter of Oddi is a goal during endoscopic retrograde cholangio-pancreatography. Systemically administered GTN has been shown previously to lower basal tone in the sphincter of Oddi during such procedures, although its use is limited by the development of systemic hypotension.

Topical application of S-nitroso-N-acetylcysteine to the ampulla and peri-ampullary duodenal mucosa of humans undergoing endoscopic retrograde cholangio-pancreatography caused a reduction in the frequency of sphincter contractions and in duodenal motility, without a fall in blood pressure, thus facilitating cannulation.

[0047] GNSO has been shown to inhibit DNA synthesis and to increase cGMP production by activated T lymphocytes, thus

suggesting a role in the prevention of T-cell mediated inflammation. GNSO also has a cytotoxic effect on leukemia cells. Irradiation of GNSO with visible light (340 or 545 nm) resulted in enhancement of this effect, and oxyhemoglobin, a nitric oxide scavenger, decreased it.

[0048] Because of their wide range of effects, RSNOs have great potential as therapeutic agents. However, interactions with copper, thiols, ascorbic acid, and other reducing agents influences their stability and ability to act as useful therapeutic agents.

[0049] Nitric oxide is unable to react with nucleophiles such as SH groups under oxygen-free conditions, implying that metabolites of NO oxidation, such as N_2O_3 , are the actual nitrosating agents (Wink et al., 1994; Lewis et al., 1994; Kharitonov et al., 1995; Williams, 1997). However, considering the low concentration and short life span of NO *in vivo* ($t_{1/2} \sim 0.1$ s) (Williams, 2001), the third order reaction of NO with oxygen (Moncada et al., 1993), ($k = 6 \times 10^6$ M⁻²sec⁻¹) (Wink et al., 1994; Lewis et al., 1994) seems to be too slow to account for any detectable amount of circulating S-nitrosothiols. The high instability of N_2O_3 in aqueous solution (Stamler et al., 1992) further supports this notion.

[0050] Recently, these apparent theoretical constraints have been resolved by the demonstration of remarkable

properties of NO in multi-phase systems (Rafikova et al., 2002; Nedospasov et al., 2000). NO and O₂ are both hydrophobic molecules that are more soluble in lipophilic solvents than in water, with a partition coefficient Q in each case >>1. Areas of high hydrophobicity act as a sponge to increase the local concentration of NO and O₂ by sequestering them from the surrounding aqueous phase. Under aerobic conditions, high local concentrations of NO and O₂ in the hydrophobic phase, e.g., within lipid membranes, can significantly accelerate NO oxidation and N₂O₃ formation (Nedospasov et al., 2000; Liu et al., 1998; Goss et al., 1999). This process, known as micellar catalysis, must play a crucial role in NO biochemistry (see Figure 1).

[0051] Body fluids and tissues represent a complex multiphase system where distribution of free NO and O₂ and the rate of formation of nitrosating species (N₂O₃) should depend on the size and geometry of the hydrophobic phases. Other than lipid membranes, there are many substances that form hydrophobic micelles *in vivo*, e.g., cholesterol, fatty acids, and hydrophobic cores of protein molecules, with a different Q in each case. Recently, it has been demonstrated that micellar catalysis of NO oxidation is mediated by serum albumin, which has a Q_{NO}~120 (Nedospasov et al., 2000). Albumin is the most abundant transport and depot protein in

the circulation. Since the concentration of albumin in plasma is large (~800 μ M), its hydrophobic core serves as a major absorber of free NO and a catalyst of N_2O_3 (Nedospasov et al., 2000). This view is further supported by data which demonstrate that albumin is an efficient catalyst of nitrosation of its own Cys³⁴ and Trp²³⁰ (Nedospasov et al., 2000) as well as circulating low molecular weight RSH (Rafikova et al., 2002).

[0052] Since S-nitrosothiols compounds are potent vasodilators and antiplatelet agents, they are considered to be promising therapeutics for a variety of acute and chronic conditions (Hogg, 2000; Al-Sa'doni et al., 2000; Richardson et al., 2002). Understanding the principles of S-nitrosothiol formation *in vivo* makes it possible to design new approaches to regulate blood flow and clotting and other processes dependent upon nitric oxide. One such an approach is the use of a synthetic hydrophobic phase, such as perfluorocarbons, for the catalysis of NO oxidation and nitrosothiols formation.

[0053] Perfluorocarbons are chemically inert, synthetic hydrophobic molecules that possess a unique capability for dissolving a variety of gases, including O_2 , CO_2 , and NO. Most therapeutic uses of fluorocarbons are related to the remarkable oxygen-carrying capacity of these compounds. One commercial biomedical perfluorocarbon emulsion, Fluosol (Green

Cross Corp., Osaka, Japan) is presently used as a gas carrier to oxygenate the myocardium during percutaneous transluminal coronary angioplasty. Perfluorocarbon emulsions have also been used in diagnostic applications such as imaging.

SUMMARY OF THE INVENTION

[0054] It is an object of the present invention to overcome the aforesaid deficiencies in the prior art.

[0055] It is another object of the present invention to control nitric oxide metabolism by administering perfluorocarbons.

[0056] It is yet another object of the present invention to inhibit nitric oxide activity by administering perfluorocarbons and treat conditions associated with nitrosative stress.

[0057] It is yet another object of the present invention to form S-nitrosothiols *in vivo* for treating a variety of conditions.

[0058] It is a further object of the present invention to catalyze NO oxidation *in vivo*.

[0059] It is another object of the present invention to potentiate NO donors formation by administering perfluorocarbons, either alone or in combination with a nucleophile.

[0060] It is a further object of the present invention to treat acute hypotension and vasoplegia, particularly when associated with septic shock.

[0061] It is another object of the present invention to induce vasorelaxation.

[0062] It is still a further object of the present invention to provide antiplatelet effects.

[0063] It is another object of the present invention to protect against myocardial ischemic-reperfusion injury.

[0064] It is a further object of the present invention to prevent and treat osteoporosis.

[0065] It is another object of the present invention to treat diseases and conditions which are affected by the production or inhibition of nitric oxide, including, but not limited to, cardiovascular diseases, respiratory diseases, microbial infections, viral infections, and gastric diseases.

[0066] It is a further object of the present invention to treat diseases and conditions which are mediated by cGMP.

[0067] It is another object of the present invention to generate S-nitrosothiols *in vivo* to treat diseases and conditions which are mediated by cGMP.

[0068] It is still another object of the present invention to generate S-nitrosothiols *in vivo* to treat the cardiovascular system, to influence platelet function, to

inhibit vasospasm and ischemia/reperfusion injury, to inhibit pre-eclampsia, to treat respiratory disease by inhibiting broncho-constrictor effects, to reduce frequency in sphincter contractions and improve duodenal motility, to inhibit DNA synthesis and to increase cGMP production by activated T lymphocytes.

[0069] It is another object of the present invention to generate S-nitrosothiols *in vivo* to treat bacterial and viral infections, including the common cold and HIV.

[0070] According to the present invention, perfluorocarbons are used to regulate nitric oxide bioactivity. By controlling nitric oxide bioactivity by administering perfluorocarbons, to avoid either an overabundance or deficiency of nitric oxide, it is possible to control a great number of physiological processes and thus treat or prevent many disease conditions. For example, sequestration of circulating nitric oxide by about 1% w/v perfluorocarbons leads to rapid and potent increase of systemic blood pressure. This property of fluorocarbons can be used to treat acute hypotension and vasoplegia, particularly in septic shock. Alternatively, administration of small amounts (less than about 0.5% w/v) of perfluorocarbons potentiates nitric oxide activity due to acceleration of NO oxidation and NO-donor formation.

[0071] Since nitric oxide contributes to the pathogenesis of a number of diseases, including hypertension, atherosclerosis, and diabetes, control of nitric oxide bioactivity enables one to treat these diseases.

[0072] To inhibit nitric oxide activity, perfluorocarbons are administered in amounts of at least about 0.5% w/v of total blood, or more. To potentiate nitric oxide activity, perfluorocarbons are administered in amount of less than about 0.5% w/v of blood. To enhance nitric oxide even further, a nucleophile is administered in conjunction with or shortly before or after administration of the perfluorocarbon.

[0073] For example, sequestration of circulating nitric oxide by ~0.7% w/v perfluorocarbons leads to rapid and potent increase of systemic blood pressure (Fig. 4). This property of perfluorocarbons can be used to treat acute hypotension and vasoplegia, particularly in septic shock.

[0074] Thus, according to the present invention, any disease or condition caused by an excess or a deficiency of nitric oxide can be treated by administering the appropriate amount of perfluorocarbon.

[0075] Regulating the activity of NO by administering perfluorocarbons is used to control vascular tone and blood pressure, including pregnancy-induced hypertension

(preeclampsia). This is important both for humans and livestock.

[0076] Since nitric oxide is a transduction mechanism for steroid hormones, regulation of nitric oxide oxidation by administering perfluorocarbons can be used to regulate estrogen and/or progesterone-dependent steps in reproduction and health of females, including ovulation, implantation, menstruation, climacterium, etc.

[0077] Nitric oxide is a potent inhibitor of blood coagulation, and potentiation of nitric oxide activity by administering perfluorocarbons can be used to prevent clotting in the circulation.

[0078] Regulating nitric oxide oxidation by administering perfluorocarbons can be used to reduce the incidence and severity of cardiovascular disease, including pathophysiological changes in blood vessels in cardiovascular diseases associated with hypertension and atherosclerosis. Administration of perfluorocarbons to postmenopausal women in addition to or instead of hormone replacement therapy can be used to prevent cardiovascular diseases.

[0079] Administering perfluorocarbons to regulate nitric oxide activity can also be used to treat osteoporosis, Paget bone disease, and rheumatoid arthritis by regulating

perturbations in the interactions between local and systemic bone-remodeling regulatory pathways.

BRIEF DESCRIPTION OF THE DRAWINGS

[0080] Figure 1 illustrates micellar catalysis of NO oxidation and S-nitrosation.

[0081] Figure 2 shows the perfluorocarbon components of Perftoran, perfluorodecaline, and perfluoromethylcyclopiperidine.

[0082] Figure 3A shows a representative NO-electrode tracing of Cu²⁺-dependent NO production after an addition of 1 mM GSH to 1% v/v Perftoran (PFC tracing) or control (Tris-HCl buffer [80 mM, pH 7.9] (buffer tracing). Bolus NO (100 μM water solution) was added to Perftoran or buffer prior to GSH addition. As soon as the concentration of NO in each probe dropped to less than 1 μM, as detected electrochemically, GSH was added.

[0083] Figure 3B shows perfluorocarbon-mediated generation of GS-NO as a function of the volume of the hydrophobic phase (% v/v Perftoran). Low molecular weight S-nitrosothiols were determined using CuCl₂ to displace NO from thio residues followed by electrochemical detection of released NO (Rafikova et al., 2002).

[0084] Figure 4A shows the effect of the "high dose" (7% v/v) of perfluorocarbon on mean arterial pressure of anesthetized rats.

[0085] Figure 4B shows the relation of perfluorocarbon-mediated hemodynamic effects to NO.

[0086] Figure 5 shows the effect of the administration of the "low dose" (1% v/v) of perfluorocarbon on mean arterial pressure.

[0087] Figure 6A shows the combined effect of perfluorocarbons and thiols (L-cysteine) on mean arterial pressure. Figure 6B shows the change in plasma low molecular weight nitrosothiols in response to perfluorocarbons and cysteine.

[0088] Figure 7 illustrates the cascade mechanism of nitrosothiols formation mediated by α -lipoic acid.

[0089] Figure 8 demonstrates the simulating effect of α -dihydrolipoic acid on the formation of nitrosothiols as well as vasorelaxation in the presence of perfluorocarbons.

[0090] Figure 9 illustrates small molecule nucleophiles that can be coadministered with perfluorocarbons to potentiate the action of the perfluorocarbons.

[0091] Figures 10A and 10B demonstrate the use of perfluorocarbons in treating endotoxic shock.

DETAILED DESCRIPTION OF THE INVENTION

[0092] The present invention provides a method for regulating nitric oxide oxidation by administering perfluorocarbons. Perfluorocarbons are powerful modulators of nitric oxide activity in mammals, including humans, and thus can be used to treat any disease or disorder that is affected by nitric oxide activity and/or the level of nitric oxide in the organism.

[0093] At low levels, when perfluorocarbons are used as a catalyst of nitric oxide oxidation, the effect of the perfluorocarbons is greatly enhanced by the addition of nucleophiles, such as natural or synthetic thiols, which react with the oxidized form of NO and subsequently donate nitric oxide. These nucleophiles can be administered contemporaneously with the perfluorocarbons, before administration of the perfluorocarbons, or after administration of the perfluorocarbons.

[0094] Regulating nitric oxide oxidation can be used for treating the following conditions, which are listed for purposes of illustration and not for limitation:

[0095] Cardiovascular conditions, including hypertension, angina, hypotension, atherosclerosis, preeclampsia (pregnancy induced hypertension, toxemia, eclampsia, HELP syndrome),

regulation of vascular conductance, regulation of blood flow, regulation of blood pressure, and myocardial ischemia;

[0096] Gastrointestinal disease, to alter motility and to treat pyloric stenosis;

[0097] Lung functions including asthma, treatment of premature babies to increase lung function, and pulmonary hypertension;

[0098] Inflammation, autoimmune and immune diseases and conditions including acute inflammation, arthritis, resistance to infection, cancer, lupus, anaphylactic reactions, and allograft rejections;

[0099] Central nervous system conditions, including behavior, epilepsy, Alzheimer's disease, stroke, and growth hormone disorders such as acromegaly;

[0100] Diseases of the pancreas, including diabetes;

[0101] Female reproductive systems or problems, including ovulation, implantation/*in vitro* fertilization, premenstrual syndrome, dysmenorrhea, uterine contractile disorders, premature labor, cervical dilation, contraception, symptoms of menopause, osteoporosis, endocrine disorder, and hormone replacement;

[0102] Male reproductive problems such as impotence, penile erection, symptoms of male menopause, endocrine disorders, osteoporosis, and prostate hypertrophy;

[0103] Bladder and kidney problems, including incontinence, renal arterial stenosis, hypertension;

[0104] Dermatological problems, including topical hair loss, eczema, autoimmune skin disease, and psoriasis.

Additionally, perfluorocarbons are administered to produce S-nitrosothiols *in vivo* to treat bacterial and viral infections including the common cold and HIV infection.

[0105] In the studies described hereinbelow, the perfluorocarbon used was a clinically approved perfluorocarbon emulsion, Perftoran (Perftoran, Inc, Russia). Perftoran contains 14% w/v/ perfluorodecaline and 6% w/v perfluoromethylcyclopiperidine that form ~0.07 micrometer micelles which are stabilized by a poloxamer-type surfactant (Sahn, 1999). For convenience, the concentration of perfluorocarbon in all experiments is indicated as % of Perftoran emulsion in the total volume of blood or buffer (v/v).

Perfluorocarbons (PFC) as NO absorbent and catalyst of NO oxidation

[0106] The maximum acceleration value (H) of NO oxidation by the hydrophobic phase of Perftoran was calculated by first determining the partition coefficient Q_{NO} ($Q_{NO} = [NO]_{PFC}/[NO]_{H2O}$) for a PFC/H₂O system. A Clark-type NO electrode (WPI Inc.) was used to monitor changes in the NO concentration in water upon

addition of PFC in an air-tight oxygen-free device. Oxygen was removed from water and Perftoran prior to the experiment by bubbling each of Perftoran and water with argon for 12 hours. The $Q_{NO}(PFC/H_2O)$ was found to be approximately 200. $Q_{O_2}(PFC/H_2O)$ was found previously to be about 20 (Gervits, 1994).

[0107] For the tri-molecular reaction of N_2O_3 formation, $H = Q_{NO}^2 \times Q_{O_2} = 200^2 \times 10 = 4 \times 10^5$. This result suggests that even a small amount (1% v/v) of perfluorocarbon emulsion in mammalian blood should sequester a significant amount of circulating NO. At the same time it should accelerate the production of vasoactive NO metabolites, such as S-nitrosothiols, due to micellar catalysis of NO oxidation. These S-nitrosothiols can be created *in vivo* for treatment of a wide variety of diseases and conditions, as described above.

Stimulating effect of perfluorocarbon on the formation of S-nitrosothiols.

[0108] A representative experiment, illustrated in figure 3A, shows that 1% v/v perfluorocarbon that was saturated with NO (0.5 μ M final concentration) increases the rate of S-nitroso-glutathione (GS-NO) formation more than 20-fold *in vitro*. Under similar conditions, perfluorocarbon was also shown to accelerate S-NO-cysteine, S-NO-homocysteine, and nitrite formation. The relative stimulating effect of

perfluorocarbons on GSH nitrosation was even greater at the lower, close to *in vivo*, concentration of NO. Importantly, the efficiency of perfluorocarbon-mediated S-nitrosation also depended upon the amount of perfluorocarbon in the probe, as shown in Figure 3B. The resonance-like maximum was observed at about 1% v/v Perftoran. Larger or smaller amounts of perfluorocarbon were progressively less efficient. This observation is readily explained in terms of micellar catalysis of NO oxidation, when the relatively small "optimal" volume of the hydrophobic phase (in this case perfluorocarbon) generates the maximum acceleration of the reaction (Rafikova et al., 2002; Beda et al., 1999). These results suggest that by using various amounts of perfluorocarbon together with low molecular weight nucleophiles, one can regulate the level of endogenous vasoactive low molecular weight S-nitrosothiols. Notably, uncoupling NO-donors formation from overall NO synthesis not only provides a new way to control blood pressure and organ perfusion, but also creates a unique opportunity to relieve the nitrosative stress (excessive nitration and nitrosation of proteins and nucleic acids) during inflammation (Poli, 2002; Beckman et al., 1996; Beckman et al., 1994). Thus, perfluorocarbons can be administered to regulate blood pressure and blood clotting. Studies in humans

and animals have shown that the body retention half-life of Perftoran is about 48 hours (Lowe, 1999).

Perfluorocarbon as a powerful NO sink in vivo and catalyst of S-nitrosothiols

[0109] The effect of Perftoran on mean arterial pressure (MAP) in rats was studied to evaluate the ability of perfluorocarbon micelles to absorb circulating NO *in vivo*, and the physiological role of this phenomenon. Intravenous perfusion of 5 ml/kg (~7% v/v) Perftoran was characterized by extensive hypertension, as shown in Figure 4A. It should be noted that the solution of perfluorocarbon that was used was isooncotic. Also, the amount of Perftoran was only about 0.7% w/v of total blood, and the rate of its infusion was slow (0.2 ml/min). This rules out the possibility that perfluorocarbon affected mean arterial pressure by changing the osmotic status.

[0110] In Figure 4A, infusion of 5 ml/kg Perftoran produced a steep increase in blood pressure (11± 4 mm Hg), followed by a gradual asymptotic recovery. Delivery of Perftoran or saline intravenously was at a constant rate of 0.2 ml/min with an electronic perfusator. The mean ± SE was obtained from 111 independent experiments.

[0111] To confirm that the increase of blood pressure by perfluorocarbons was due to NO sequestration, the following

experiment was conducted, as illustrated in Figure 4B. First, the experimental group of animals received a non-selective NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg, intraperitoneally) to deplete the vasculature of endogenous NO, thus causing a characteristic increase of mean arterial pressure, as shown in Figure 4B. Perftoran failed to increase mean arterial pressure further, as shown in the right panel of Figure 4B, suggesting that endogenous NO was essential for perfluorocarbon-induced vasoconstriction. Furthermore, the infusion of 1 mg/kg potassium nitrite about 90 minutes after the L-NAME treatment partially restored mean arterial pressure, as shown in Figure 4B. In this case, administration of Perftoran elevated mean arterial pressure back to the original L-NAME level, as shown in Figure 4B. The nitrite was readily reduced to NO *in vivo*, which explains its vasorelaxation effect. The ability of Perftoran to effectively reverse the nitrite effect on mean arterial pressure confirmed its mode of action as a powerful NO sink.

[0112] It was noted that about 30 minutes after infusion of Perftoran, nitrite-treated rats exhibited a much stronger decrease of mean arterial pressure (mean arterial pressure dropped to the original baseline level) than the control group without Perftoran (mean arterial pressure remained about 24 mmHg above the baseline, as shown in Figure 4B).

[0113] Also, a modest but reproducible delayed decrease of mean arterial pressure by perfluorocarbons was observed in the experiment without nitrite and L-NAME, shown in Figure 4A. These results can be interpreted as that, during the first phase of action, perfluorocarbon rapidly adsorbs NO, causing the initial increase of mean arterial pressure. However, since the local concentration of NO and O₂ in perfluorocarbon micelles becomes high, the nitrosating species (N₂O₃) form as a result of accelerated NO oxidation. This process, in turn, leads to vasoactive S-nitrosothiols formulation, shown in Figure 3, and vasorelaxation. The experiment shown in Figure 5 supports this hypothesis. Here, animals received 10 times less Perftoran intravenously than in the experiment shown in Figure 4. Although the initial jump of mean arterial pressure was not as dramatic, a subsequent drop in mean arterial pressure was significantly more rapid and pronounced (compare Figure 5 with Figure 4A). The reasonable explanation for such a reverse dose-dependent effect of perfluorocarbon on blood pressure originates from the micellar catalytic nature of PCF/NO interactions. As mentioned above, the theory of micellar catalysis states that there should be an optimal volume of a hydrophobic phase, in this case perfluorocarbon, to have a maximum stimulating effect on the reaction, in this case oxidation of NO and formation of nitrosating species

(Rafikova et al., 2002; Beda, et al., 1999). Indeed, according to Figure 3B, about 1 v/v% Perftoran generated the highest yield of S-nitrosothiols. Larger or smaller amounts of perfluorocarbon are progressively less efficient. Assuming that Perftoran catalyzes formation of S-nitrosothiols *in vivo*, this dose-dependent vasodilatory effect is expected to reflect the dose-dependent curve of Perftoran-mediated S-nitrosothiols formation *in vitro* (Figure 3B). Accordingly, 1% v/v / perfluorocarbon (Figure 5) relaxes vascular tone more efficiently than 7% v/v perfluorocarbon (Figure 4A).

[0114] To directly demonstrate the ability of perfluorocarbons to catalyze S-nitrosothiol formation *in vivo* and to confirm its relation to perfluorocarbon-induced vasorelaxation, measurements of mean arterial pressure were conducted in parallel with direct monitoring low molecular weight-S-nitrosothiols in plasma before and after Perftoran administration, as shown in Figure 6. Plasma low molecular weight-S-nitrosothiols were determined using CuCl₂ to displace NO from thiol residues followed by electrochemical detection of released NO (Rafikova et al., 2002). The concentration of CuCl₂, 40 μ M, was selected to decompose predominantly low molecular weight S-nitrosothiols. The relatively stable high molecular weight S-nitrosothiols (e.g., S-NO-albumin) were mostly resistant to such low concentrations of Cu²⁺

(unpublished observation). As Figure 6B shows, the level of plasma low molecular weight S-nitrosothiols increased by about 40 nM (about 10-fold) after 10 minutes of 1% v/v Perftoran injection, and then gradually decreased over the next hour.

[0115] To confirm that induced vasorelaxation was due to S-nitrosothiols formation, the effect of exogenous molecular weight thiols on mean arterial pressure and S-nitrosothiols formation was studied in perfluorocarbon treated animals. Without perfluorocarbon, cysteine (1 mg/kg, intravenously) had virtually no effect on plasma low molecular weight S-nitrosothiols or on blood pressure, as shown in Figure 6. However, in the PFC-pretreated rats, the same amount of cysteine caused more than 100 fold increase of plasma low molecular weight S-nitrosothiols (Figure 6B), and significant decrease of mean arterial pressure (Figure 6A). The decrease of mean arterial pressure due to the combined effect of perfluorocarbon and cysteine was much stronger than that of perfluorocarbon alone (compare Figure 5 with Figure 6A). Similar vasorelaxation effects and stimulation of S-nitrosothiols formation were observed when another low molecular weight thiol, GSH, was administered to perfluorocarbon pretreated rats instead of cysteine. Taken together, the results obtained demonstrate that perfluorocarbons can preserve otherwise unstable circulating

NO in a form of more stable vasoactive low molecular weight S-nitrosothiols, which explains the hemodynamic effects of perfluorocarbons.

[0116] The unique ability of perfluorocarbons to control NO bioactivity can be used to regulate blood pressure and clotting and other NO-dependent processes with minimum side effects that are normally associated with activation/inhibition of NOS. Thus, in order to increase blood flow one can administer >>1% v/v perfluorocarbons to rapidly sequester free circulating NO without compensatory production of S-nitrosothiols due to inefficiency of micellar catalysis of NO oxidation. On the other hand, to decrease blood pressure to a steady level, one can administer about 0.5% or less v/v perfluorocarbon with various low molecular weight nucleophiles. Under these conditions, a relatively small increase in plasma nucleophile, such as low molecular weight thiols, would have a great stimulating effect on vasodilatory S-nitrosothiols production, as shown in Figures 6A and 6B. Since perfluorocarbons have a half-life in mammals of from about 24-48 hours, the perfluorocarbons could be administered as infrequently as several times a week.

[0117] A common cause of acute inflammation or sepsis are severe bacterial infections that are frequently associated with hospitalized and/or otherwise exhausted individuals in

which a standard host defense is compromised. Septic shock is associated with high mortality, and current therapy is mostly supportive and ineffective (Nakazawa et al., 2000; Wenzel, et al., 1996). During septic shock, systemic overproduction of NO by transient early activation of endothelial NOS (eNOS) and a subsequent hyper-induction of inducible NOS (iNOS) is the major cause of acute hypotension and poor organ perfusion (Wenzel et al., 1996; Titheradge, 1999). It also leads to excessive protein and nucleic acid nitration in tissues and body fluids, apparently because of the presence of a high level of ONOO^- , a product of the reaction between NO and O_2^- (Beckman et al., 1996; Beckman et al., 1994; Titheradge, 1999). On the other hand, activation of iNOS in white blood cells is one of the key mechanisms to combating infection (Titheradge, 1999; DeGroote et al., 1999). The unique ability of perfluorocarbons to rapidly sequester circulating NO without suppressing NOS activities makes perfluorocarbons useful in attenuating hypotension and vasoplegia during septic shock. Perfluorocarbon micelles cannot penetrate the cell membrane or diffuse into the tissue, and thus they should not significantly affect the intracellular NO levels, which is required for a potent immune defense (Titheradge, 1999; DeGroote et al., 1999). Furthermore, by decreasing the concentration of circulating NO, perfluorocarbons are expected

to subdue the ONOO^- mediated tissue damage. Finally, unlike NOS inhibitors, perfluorocarbon emulsions are not expected to cause excessive vasoconstriction, platelet aggregation, leukocyte adhesion, and many other adverse effects of systemic NO depletion (DeGroote et al., 1999) because of a compensatory production of S-nitrosothiols.

[0118] If delivered at small concentrations, i.e., less than 1% v/v, and in conjunction with low molecular weight nucleophiles, such as thiols, perfluorocarbons act as a powerful vasodilator rather than as a vasoconstrictor. This effect is due to micellar catalysis of vasoactive S-nitrosothiols formation by perfluorocarbons, as shown in Figures 3, 5, and 6. NO^+ originated from N_2O_3 in the perfluorocarbon micelles can be transferred to various low molecular weight nucleophiles, exemplified as RSH, by two possible pathways, as shown in Figure 7. Direct attack on RSH is possible at the surface of perfluorocarbon micelles or inside the micelles, if the RSH were hydrophobic enough to enter it, as shown in pathway I of Figure 7. Additionally, NO^+ can reach various RSH via a transnitrosation reaction by using a lipophilic low molecular weight "shuttle", as shown in Figure 7. One example of such an NO^+ shuttle is α -lipoic acid (α -LA), which is normally present in the circulation at low (micromolar) concentrations (Keipert, 2001). α -lipoic acid

has a highly hydrophobic tail (Figure 7A), which can readily penetrate the perfluorocarbon micelle where it is nitrosated. Since both SH groups of α -dihydro-lipoic acid are located next to each other, upon nitrosation they would almost immediately form an S-S adduct and release NO^+ . Thus, α -lipoic acid may serve as a shuttle to transfer NO^+ from the perfluorocarbon micelle interior to outside hydrophilic low molecular weight RSH thus potentiating the PFC-mediated RS-NO formation (Figure 7B). Our *in vitro* and *in vivo* results support this conclusion (Figure 8). Since low molecular weight S-nitrosothiols are potent vasodilators and anticoagulants, they are regarded as promising cardiovascular therapeutics (Hogg, 2000; Al-Sa-doni et al., 2000; Richardson, et al., 2002). However, the major drawback of available low molecular weight S-nitrosothiols is that they are very unstable in physiological vehicles. Moreover, high doses of S-nitrosothiols can be toxic and dangerously affect blood flow. Therefore, the use of stable and safe RSH, such as GSH, cysteine, and α -lipoic acid in conjunction with perfluorocarbons to achieve the same effects as that of corresponding exogenous S-nitrosothiols should be advantageous.

[0119] The unique properties of perfluorocarbons as carriers of O_2/NO and generators of S-nitrosothiols make them useful in protecting against myocardial ischemic-reperfusion

injury. Perfluorocarbon micelles are smaller than red blood cells (e.g., 0.07 micrometer for Perfotoran, Loew, 1999; Spahn, 1999), and thus should better overcome vascular occlusion to deliver O₂ to ischemic tissue. Many experiments on rabbits and dogs have demonstrated significant reduction of myocardial infarct size by intravenous Fluosol treatment (Timmermans et al., 1993; Forman et al., 1985; Rice et al.). However, subsequent Fluosol experiments with rabbits and human trials with Fluosol failed to demonstrate such a healing effect (Forman et al., 1992; Wall et al., 1994). Fluosol, the only perfluorocarbon approved in the U.S. for angioplastic procedures, has a number of disadvantages in comparison with more advanced perfluorocarbon emulsions such as Perfotoran and Oxygent, including larger particle size, lower O₂ capacity, higher viscosity, and instability (Lowe, 1999). Most importantly, previous studies have not considered the role of perfluorocarbons as modulators of NO bioactivity.

[0120] The well-established cytoprotective action of NO during perfusion-reperfusion (reviewed in Kloner et al., 1994; Hale et al., 1995) and its favorable effects on diastolic dysfunction (Rakhit et al., 2001), often associated with reperfusion injury, provide a rationale for using perfluorocarbons as NO-releasing agent in this context. It has been noted that perfluorocarbons preserve the endothelium

and prevent NO-reflow by inhibiting neutrophil function, including adherence to endothelial cells and release of toxic substances (reviewed in Vinten-Johansen et al., 1999). The same function has been attributed to NO in many studies (Hale et al., 1995; Paulus et al., 1994). NO also plays a principal role in delaying ischemic preconditioning, the phenomenon in which a stimulus, which can be brief coronary ischemia, renders the myocardium more resistant against the subsequent sustained ischemia and reperfusion. Generally, the cardioprotective effects of NO against ischemia/reperfusion injury may be achieved directly or indirectly through its well-known biological actions such as coronary vasodilation, reduction of myocardial O₂ consumption, and opening of KATP channels (Heard et al., 2000; Yin et al., 1997). Recently, NO releasing substances have been also shown to suppress restenosis after transluminal coronary angioplasty (Miyoshi et al., 1994; Sasaki et al., 2000).

[0121] A study was conducted to demonstrate the use of perfluorocarbons in treating endotoxic shock in rats. Male Wistar rats (15 animals per group) weighting 250-300 grams were anesthetized intraperitoneally with 1.2 mg/kg of urethane. The right femoral artery was cannulated with PE50 tubing and connected to a PowerLab pressure transducer for

measuring arterial blood pressure. PE50 catheters were placed into the right femoral vein for infusion of various agents.

[0122] Sepsis was induced intravenously by a bolus of endotoxin from *Escherichia coli* LPS, 50 mg/kg. The results shown in Figure 10A demonstrate that a single bolus infusion of perfluorocarbon, 7% v/v, 0.2 ml/min, thirty minutes after administration of LPS improves the hemodynamic by preventing loss of blood pressure. The control treatment was no treatment, and HES was the standard starch treatment. In Figure 10A, MAP is mean arterial pressure.

[0123] As shown in figure 10B, the survival of the animals after 24 hours was increased six-fold in comparison with control and HE S groups, respectively.

[0124] In contrast with its cardioprotective actions, NO in larger quantities, such as those normally associated with inflammation and generation of deleterious ONOO⁻, has also been implicated in promulgating coronary injury (Maffia et al., 2002). For example, L-NAME NOS inhibitor reduced infarct size in an *in situ* rabbit model (Min et al., 2001), and this cardioprotective effect can be reversed by co-administration of L- but not D-arginine, confirming involvement of the L-arginine NO pathway (Bolli, 2001). In a majority of experiments, however, L-arginine, NO gas, and NO-donors have been shown to protect against reperfusion injury to various

degrees (Woolfson et al., 1995; Schulz et al., 1995; Johnson et al., 1991). Evidence suggests that the protective effect, in large part, is due to attenuation of neutrophil-endothelial cell interactions (Hale et al., 1995; Siegfried et al., 1992). Thus, NO levels should be finely balanced in the ischemic tissue to exert its cardioprotection, since a deficiency as well as an excess of NO can lead to further tissue damage. The use of conventional NO-donors has always been associated with two major problems: instability of most such chemicals in physiological vehicles, and their toxicity (Richardson et al., 2002). In this regard, perfluorocarbons/nucleophiles present a much safer pharmacological tool that allows for a sustained and finely regulated delivery of endogenous NO to the ischemic tissue.

[0125] To achieve maximum cardioprotection, specific iNOS inhibitors such as S-methylisothiourea or aminoguanidine can be administered together with the nucleophile to minimize excessive production of NO in the vicinity of injury and to spare the regional nitrosative stress. In this case, the major source of NO in the ischemic heart is from S-nitrosothiols that were endogenously formed in perfluorocarbon micelles far from the ischemic zone. As noted previously, low molecular weight S-nitrosothiols have a greater potency than free NO due to their higher stability and specific

intracellular transport (Rafikova et al., 2002; Hogg, 2000). At the same time, S-nitrosothiols cannot directly produce ONOO⁻ and other reactive nitrogen species, thus eliminating the major toxic effects of NO. Finally, the advantage of endogenous low molecular weight S-nitrosothiols is that their levels can be finely and safely regulated simply by changing the concentration and combination of exogenous thiols (Figure 6).

[0126] Ischemic preconditioning is an important aspect of cardioprotection that involves NO and thus can be treated with perfluorocarbon/RSH. Originally described as an immediate adaptation of the heart to brief coronary occlusion (Murry, et al., 1986), ischemic preconditioning was subsequently found to be a biphasic phenomenon, with the early phase of protection that develops within minutes from the initial ischemic insult and lasts 2 to 3 hours, and a late (or delayed) phase that becomes apparent 12 to 24 hours later and lasts for 3 to 4 days (Pabla et al., 1996; Bolli, 2001). Unlike the early phase, the late phase of ischemic preconditioning protects not only against myocardial necrosis and arrhythmias, but also against myocardial stunning (Kuzuya et al., 1993), suggesting that it may have greater clinical relevance. Many independent studies have shown that NO is not only necessary, but also sufficient to induce late ischemic preconditioning (reviewed

in Bolli, 2000; Maffia et al., 2002). Furthermore, the critical role of endogenous and exogenous NO in extension of both early and late phases of ischemic preconditioning have been well documented (Maffia et al., 2002; Marber et al., 1993; Bolli, 1996). Thus perfluorocarbon/RSH can be used to produce endogenous low molecular weight S-nitrosothiols for triggering and extending the late phase of ischemic preconditioning against myocardial infarction. The advantage of using endogenous low molecular weight S-nitrosothiols is twofold: 1. S-nitrosothiols are more efficient than NO itself, as discussed above, and 2. unlike NO, S-nitrosothiols cannot sequester oxygen free radicals and form detrimental ONOO^- . This is important not only for protection against nitrosative stress and tissue damage by ONOO^- , but also for better activation of preconditioning, since the presence of free oxygen radicals has been shown to be essential for ischemic preconditioning cardioprotection (Kuzuya et al., 1993; Xi et al., 2000).

[0127] Thus, perfluorocarbon emulsions can be used as synthetic regulators of NO bioactivity in animals, particularly in acute cardiovascular conditions. This is based upon the micellar catalysis of NO oxidation and vasoactive S-nitrosothiols formation that has been established

in vitro as well as in vivo (Rafikova et al., 2002; Nedospasov et al., 2000).

[0128] The perfluorocarbons are colorless liquids with low viscosity and densities ranging generally from about 1.8 to about 2.0 g/mL. Perfluorocarbons are immiscible with water and with most organic solvents. Therefore, for intravenous administration, the perfluorocarbons must be emulsified with aqueous solutions of electrolytes and buffers, also containing surfactants and oncotic (colloid-osmotic) components. Emulsification is achieved by ultrasonic vibration (sonication) followed by dialysis for removing any traces of toxic fluoride anions formed during sonication, or by high-pressure homogenization, which is more appropriate for large-scale manufacture of emulsions.

[0129] One example of a perfluorocarbon emulsion is Fluosol, manufactured in Japan by Green Cross Corporation. Fluosol is a 10% emulsion in injectable water made by combining three separate solutions prior to emulsification and administration as follows:

Solution 1:

Perfluorodecalin	14.0 % w/v
Perfluoro-tripropylamine	6.0
Pluronic-F-68	2.7
Yolk phospholipids	0.4

Glycerol 0.8

Solution 2:

Potassium chloride 0.034

Sodium hydrogen carbonate 0.210

Solution 3:

Sodium chloride 0.60

Calcium chloride 0.028

Magnesium chloride 0.020

Glucose 0.180

Hydroxyethyl starch 3.0

[0130] Other examples of a perfluorocarbon emulsion are Perftoran, manufactured in Russia by Perftoran Inc, and Oxygent, which is made in the U.S. by Alliance Pharmaceuticals Inc.

[0131] Other perfluorocarbon emulsions are prepared by starting with pure perfluoro precursors and reacting them with another compound to yield a pure substance. For the optimal trade-off between low volatility, leading to too high retention times in the body, and too high volatility, leading to possible circulation problems, the range of molecular weights of the perfluorocarbons should lie within the limits of about 400-700 Daltons. The optimal particle size of the perfluorocarbon micelles is from about 25 nm to about 1 micron. The surfactants used provide emulsions that have a

long shelf life at room temperature in the final sterile emulsion.

[0132] The perfluorocarbons of the present invention must be substantially non-toxic and not be significantly metabolized *in vivo*. They should also be non-immunogenic.

[0133] Representative perfluorocarbons which can be used in the present invention to regulate nitric oxide include perfluorinated hydrocarbons such as perfluorinated decalin, perfluorinated adamantine; halogen derivatives such as octyl bromide; tertiary amines such as perfluorinated tributylamine or perfluorinated tripropylamine; nitrogen-containing heterocycles such as perfluorinated N-methyl isoquinoline, perfluorinated N-(4-methylcyclohexyl)-piperidine; and oxygen-containing heterocycles such as perfluorinated 2-n-butyl-tetrahydrofuran. Other examples of highly fluorinated compounds which can be used in the present invention are perfluorooctyl ethane, perfluorohexylethane, 1,2-bis(perfluorobutyl)ethane, and 1,2bis(perfluoroethyl)ethene. Perfluorocarbons are described in, for example, U.S. Patents Nos. 3,962,439; 3,493,581; 4,110,474; 4,186,253; 4,187,252; 4,252,827; 4,423,077; 4,443,480; 4,534,978; and 4,542,147, the entire contents of each of which are hereby incorporated by reference. For the purposes of the present application, the

term "perfluorocarbons" will include all of these types of compounds, as well as mixtures thereof.

[0134] The perfluorocarbons generally comprise up to about 70-75% by volume of the emulsions used in the present invention. Preferably, the emulsions of the present invention comprise from about 10% to about 75% by volume of perfluorocarbons, with about 0.5 to about 7.5 % (by weight of the non-fluorocarbon volume) of surfactant.

[0135] Surfactants for use in the perfluorocarbon emulsions of the present invention include any of the known anionic, cationic, nonionic, and zwitterionic surfactants. Combinations of surfactants can be used in the emulsions, the most important criterion being that the surfactants are non-toxic. Particularly useful surfactants include egg phosphatides, lecithin, and alkyl salts of oleic acid such as sodium oleate.

[0136] When the perfluorocarbon is used in low concentrations as a nitric oxide oxidation catalyst, it may be administered with a low molecular weight nucleophile, which greatly enhances the effect of the perfluorocarbon.

[0137] The nucleophiles are converted into nitric oxide donors *in vivo* upon reaction with the oxidized NO species (e.g. N_2O_3) inside, or at the surface of, perfluorocarbon micelles (Fig 1, Fig 7B). Nitric oxide donors exert stronger

physiological effects than free nitric oxide due to a much longer lifetime *in vivo*.

[0138] Any non-toxic nucleophile can be used in the present invention, including natural and synthetic thiols (Fig 9). Endogenous (circulating) nitric oxide generally reacts with water, forming nitrite and nitrate. However, when a nucleophile is present, the nitric oxide is converted into a nitric oxide donor that acts the same way as nitric oxide itself, only more efficiently. The nucleophiles can be administered prior to administration of perfluorocarbons, with the perfluorocarbons, or after administration of the perfluorocarbons.

[0139] The nucleophiles are preferably low molecular weight compounds, generally less than about 5,000 Daltons, or the size of a peptide of up to about 10 amino acids, so that they can be readily incorporated into the perfluorocarbon micelles when administered with the perfluorocarbon. While thiols are among the preferred nucleophiles, one of the most preferred thiols is α -lipoic acid because of its hydrophobic tail, which contains two thiol groups at the end of the tail. Other useful thiols in the present invention include, but are not limited to, L-cysteine, N-acetyl-L-cysteine, and glutathione. Other useful low molecular weight nucleophiles are tryptophan,

melatonin, serotonin, and their derivatives, and antioxidants such as α -tocopherol (Fig 9).

[0140] Other nucleophiles that can be used as nitric oxide donors are non-toxic molecules containing iodide ion, alkyl sulfides, bromide, hydroxide, alkoxides, etc. One skilled in the art can readily determine if a substrate is an appropriate nucleophile without undue experimentation.

[0141] Administration of the perfluorocarbon compounds is in a therapeutically effective amount, i.e., that amount which corrects the particular biological problem. For inhibiting nitric oxide activity, the perfluorocarbon should be administered in amounts of at least about 0.5% w/volume of blood or more. The perfluorocarbon can be administered in larger quantities, e.g. up to about 10% w/volume of blood, depending upon the condition of the patient and the particular problem to be treated. For enhancing the effects of nitric oxide, the perfluorocarbons should be administered in amounts of less than about 0.5% w/volume of blood, and generally in amounts ranging from about 0.01% up to about 0.1% w/volume of blood. These amounts will vary with the individual and the condition to be treated.

[0142] The perfluorocarbons have a half-life in mammals of about 24-48 hours, depending upon the size of the perfluorocarbon micelle. For treating chronic conditions, the

perfluorocarbons can be administered at 24-48 hour intervals, depending upon the severity of the condition and the half-life of the particular perfluorocarbon used. For treating acute conditions, of course, the perfluorocarbons can be administered once or several times, depending upon the severity of the condition and the length of time required to affect the condition.

[0143] Administration of the perfluorocarbons can be parenteral, topical, or intracavity. Intravenously may be the preferred method of parenteral administration, for example, for treatment of hypertension. Intracavity administration may be, for example, bronchial (by inhalation), cervical, uteral, or vaginal (for remedying disorders of the reproductive tract), oral (for example, held contained in a pouch or carrier for sublingual or buccal administration, or anal. Topical administration may be by incorporating the perfluorocarbon in a suitable transdermal patch, or by incorporating the perfluorocarbon in a vehicle that enhances penetration of the skin, such as DMSO.

[0144] In determining the dosages be administered, the dosage and frequency of administration is selected in relation to the pharmacological properties of the specific active ingredients. Normally, at least three dosage levels should be used. In toxicity studies in general, the highest dose should

reach a toxic level but be sublethal for most animals in the group. If possible, the lowest dose should induce a biologically demonstrable effect. These studies should be performed in parallel for each compound selected.

[0145] Additionally, the ID₅₀ level of the active ingredient in question can be one of the dosage levels selected, and the other two selected to reach a toxic level. The lowest dose that dose not exhibit a biologically demonstrable effect. The toxicology tests should be repeated using appropriate new doses calculated on the basis of the results obtained. Young, healthy mice or rats belonging to a well-defined strain are the first choice of species, and the first studies generally use the preferred route of administration. Control groups given a placebo or which are untreated are included in the tests. Tests for general toxicity, as outlined above, should normally be repeated in another non-rodent species, e.g., a rabbit or dog. Studies may also be repeated using alternate routes of administration.

[0146] Single dose toxicity tests should be conducted in such a way that signs of acute toxicity are revealed and the mode of death determined. The dosage to be administered is calculated on the basis of the results obtained in the above-mentioned toxicity tests. It may be desired not to continue studying all of the initially selected compounds. Data on

single dose toxicity, e.g., ID₅₀, the dosage at which half of the experimental animals die, is to be expressed in units of weight or volume per kg of body weight and should generally be furnished for at least two species with different modes of administration. In addition to the ID₅₀ value in rodents, it is desirable to determine the highest tolerated dose and/or lowest lethal dose for other species, i.e., dog and rabbit.

[0147] When a suitable and presumably safe dosage level has been established as outlined above, studies on the drug's chronic toxicity, its effect on reproduction, and potential mutagenicity may also be required in order to ensure that the calculated appropriate dosage range will be safe, also with regard to these hazards.

[0148] Pharmacological animal studies on pharmacokinetics revealing, e.g., absorption, distribution, biotransformation, and excretion of the active ingredient and metabolites are then performed. Using the results obtained, studies on human pharmacology are then designed. Studies of the pharmacodynamics and pharmacokinetics of the compounds in humans should be performed in healthy subjects using the routes of administration intended for clinical use, and can be repeated in patients. The dose-response relationship when different doses are given, or when several types of conjugates or combinations of conjugates and free compounds are given,

should be studied in order to elucidate the dose-response relationship (dose vs. plasma concentration vs. effect), the therapeutic range, and the optimum dose interval. Also, studies on time-effect relationship, e.g., studies into the time-course of the effect and studies on different organs in order to elucidate the desired and undesired pharmacological effects of the drug, in particular on other vital organ systems, should be performed.

[0149] The compounds of the present invention are then ready for clinical trials to compare the efficacy of the compounds to existing therapy. A dose-response relationship to therapeutic effect and for side effects can be more finely established at this point. The amount of compounds of the present invention to be administered to any given patient must be determined empirically, and will differ depending upon the condition of the patients. Relatively small amounts of the active ingredient can be administered at first, with steadily increasing dosages if no adverse effects are noted. Of course, the maximum safe toxicity dosage as determined in routine animal toxicity tests should never be exceeded.

[0150] Perfluorocarbons are used to modulate nitric oxide activity in humans and other mammals. Perfluorocarbons can be used to treat and prevent any disease or disorder that relates to an abnormal nitric oxide activity and/or the level of

nitric oxide in an animal. At low levels, when the perfluorocarbons are used as potentiators of nitric oxide activity, the effect of the perfluorocarbons is greatly enhanced by the addition of nucleophiles, particularly natural or synthetic thiols, as well as other molecules that concentrate in whole or in part into perfluorocarbon micelles, react with, and subsequently donate nitric oxide. These molecules can be administered prior to administering perfluorocarbons, simultaneously with perfluorocarbons, or in a formulation wherein the molecules are included inside the perfluorocarbon micelles during their production:

[0151] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

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WHAT IS CLAIMED IS:

1. A method for controlling metabolism of nitric oxide comprising administering to an animal in need thereof an effective amount of a perfluorocarbon.
2. The method according to claim 1 wherein the perfluorocarbon is administered in an amount of at least about 0.5% w/volume of blood to inhibit nitric oxide activity.
3. The method according to claim 1 wherein the perfluorocarbon is administered in an amount of less than about 0.5% w/volume of blood to enhance nitric oxide activity.
4. The method according to claim 3 wherein the perfluorocarbon is administered in an amount of from about 0.01% to about 0.1% w/volume of blood to enhance nitric oxide activity.
5. The method according to claim 3 wherein at least one nucleophile is administered in conjunction with the perfluorocarbon.
6. The method according to claim 5 wherein the nucleophile is a thiol or mixture of thiols.
7. The method according to claim 6 wherein the thiol is α -lipoic acid, L-cysteine, or glutathione.
8. The method according to claim 1 wherein the animal in need thereof is suffering from a disease or condition selected from the group consisting of

cardiovascular conditions, hypertension, angina, preeclampsia (pregnancy induced hypertension, toxemia, eclampsia, HELP syndrome), hypotension, atherosclerosis, regulation of vascular conductance, blood clotting and thrombosis, regulation of blood flow, regulation of blood pressure, vasoproligia, septic shock, and myocardial ischemia; gastrointestinal disease to alter motility and pyloric stenosis;

lung functions selected from the group consisting of asthma, treatment of premature babies to increase lung function, and pulmonary hypertension;

inflammation, autoimmune and immune diseases and conditions selected from the group consisting of acute inflammation, arthritis, resistance to infection, cancer, lupus, anaphylactic reactions, and allograft rejections;

central nervous system conditions selected from the group consisting of behavior, epilepsy, Alzheimer's disease, stroke, and growth hormone disorders such as acromegaly;

diseases of the pancreas;

female reproductive systems or problems selected from the group consisting of ovulation, implantation/in vitro fertilization, premenstrual syndrome, dysmenorrhea, uterine contractile disorders, premature labor, cervical dilation,

contraception, symptoms of menopause, endocrine disorder, and hormone replacement;

male reproductive problems selected from the group consisting of impotence, penile erection, symptoms of male menopause, endocrine disorders, osteoporosis, and prostate hypertrophy;

bladder and kidney problems selected from the group consisting of incontinence, renal arterial stenosis, and hypertension;

osteoporosis, Paget bone disease, and rheumatoid arthritis;

dermatological problems selected from the group consisting of topical hair loss, eczema, autoimmune skin disease, and psoriasis.

9. The method according to claim 2 comprising treating hypotension and vasoplegia.

10. The method according to claim 3 comprising treating vasorelaxation.

11. The method according to claim 3 comprising treating blood clotting and thrombosis.

12. The method according to claim 3 comprising enhancing ischemic preconditioning.

13. The method according to claim 3 comprising suppressing restenosis after angioplasty.

14. The method according to claim 3 comprising treating preeclampsia.

15. A composition comprising a perfluorocarbon and a nucleophile.

16. The composition according to claim 15 wherein the nucleophile is a thiol.

17. The composition according to claim 16 wherein the thiol is α -lipoic acid, and/or L-cysteine, and/or glutathione.

18. A method for preparing a composition for treating nitric oxide insufficiency comprising preparing or obtaining an emulsion comprising a perfluoro compound.

19. A method for preparing a composition for enhancing the effects of nitric oxide comprising preparing or obtaining an emulsion comprising a perfluoro compound.

20. The method according to claim 18 comprising adding to the emulsion a nucleophile.

21. A method for treating conditions associated with nitrosative stress comprising administering to an animal in need thereof an effective amount of a perfluorocarbon.

22. A method for regulating perturbations in interactions between local and systemic bond-remodeling regulatory pathways comprising administering to an animal in

need thereof an effective amount of a perfluorocarbon and an optional nucleophile.

23. A method for generating S-nitrosothiols *in vivo* comprising administering to an animal in need thereof an effective amount of a perfluorocarbon and an optional nucleophile.

24. A method for treating microbial or viral infections comprising administering to an animal in need thereof an effective amount of a perfluorocarbon and an optional nucleophile.

25. The method according to claim 24 wherein the viral infections are selected from the group consisting of rhinoviral infections and retroviral infections.

26. A method for treating leukemia comprising administering an effective amount of a perfluorocarbon to a patient in need thereof.

27. The method according to claim 26 wherein the patient is also irradiated with light of a visible wavelength.

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FIG. 1

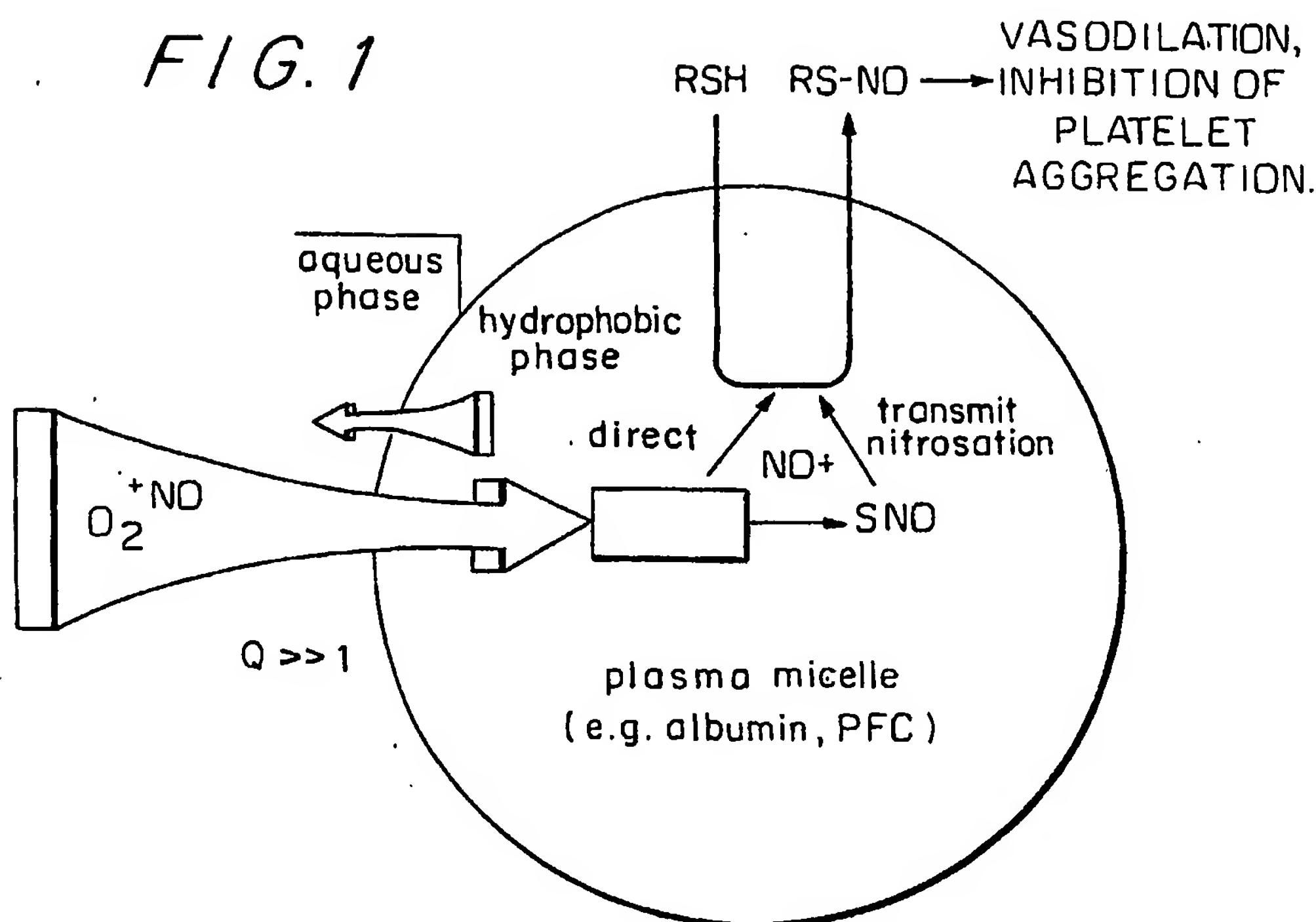
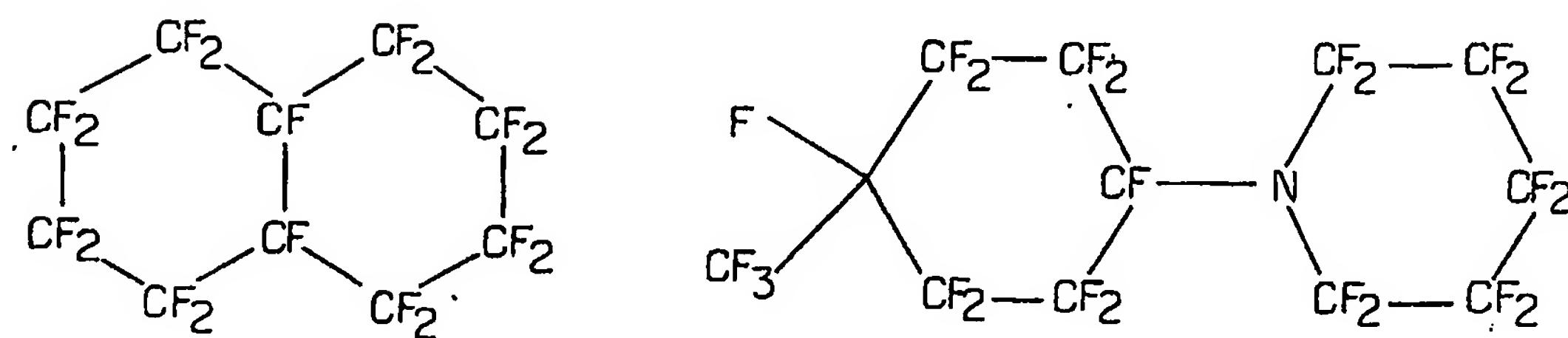
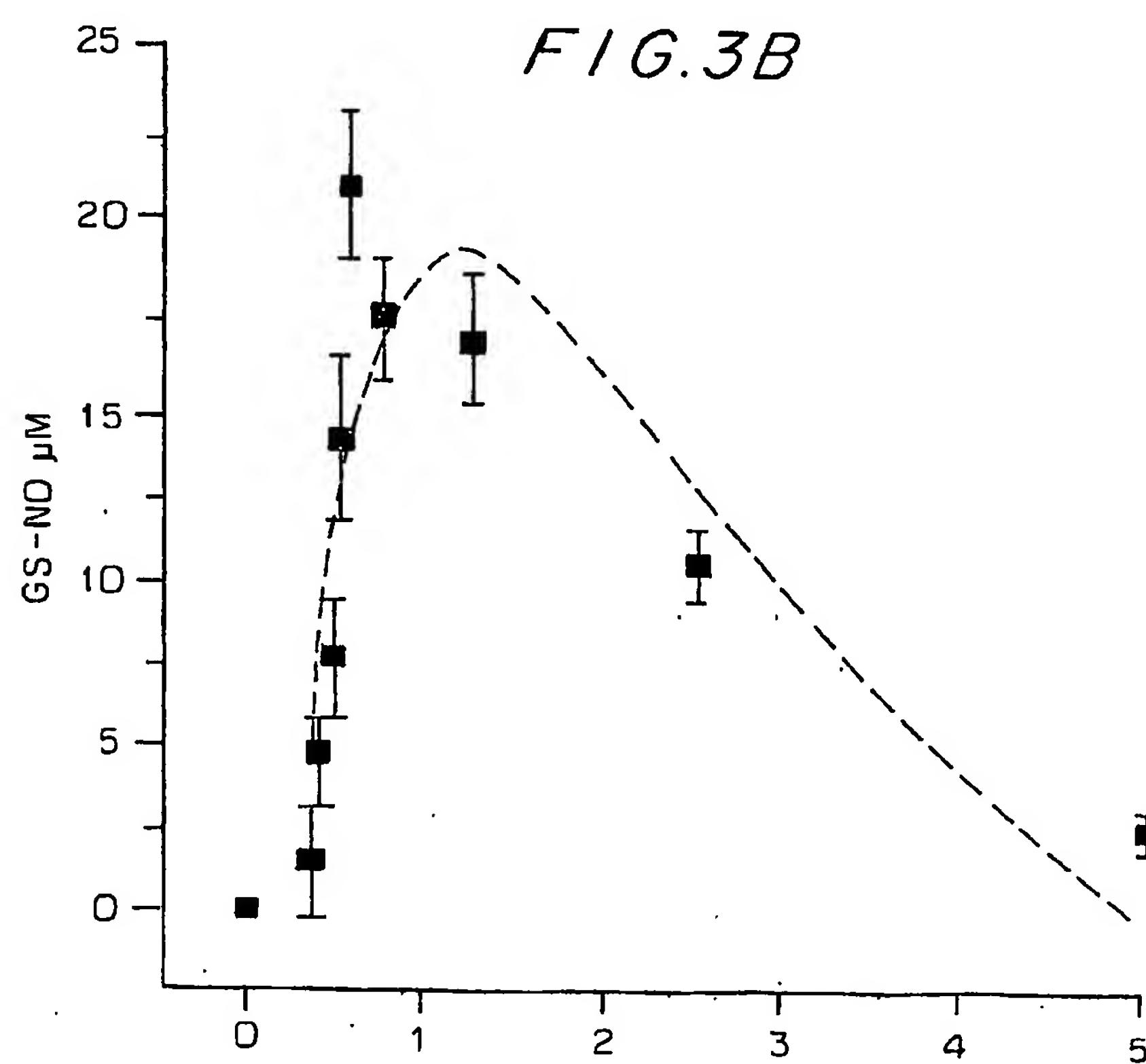
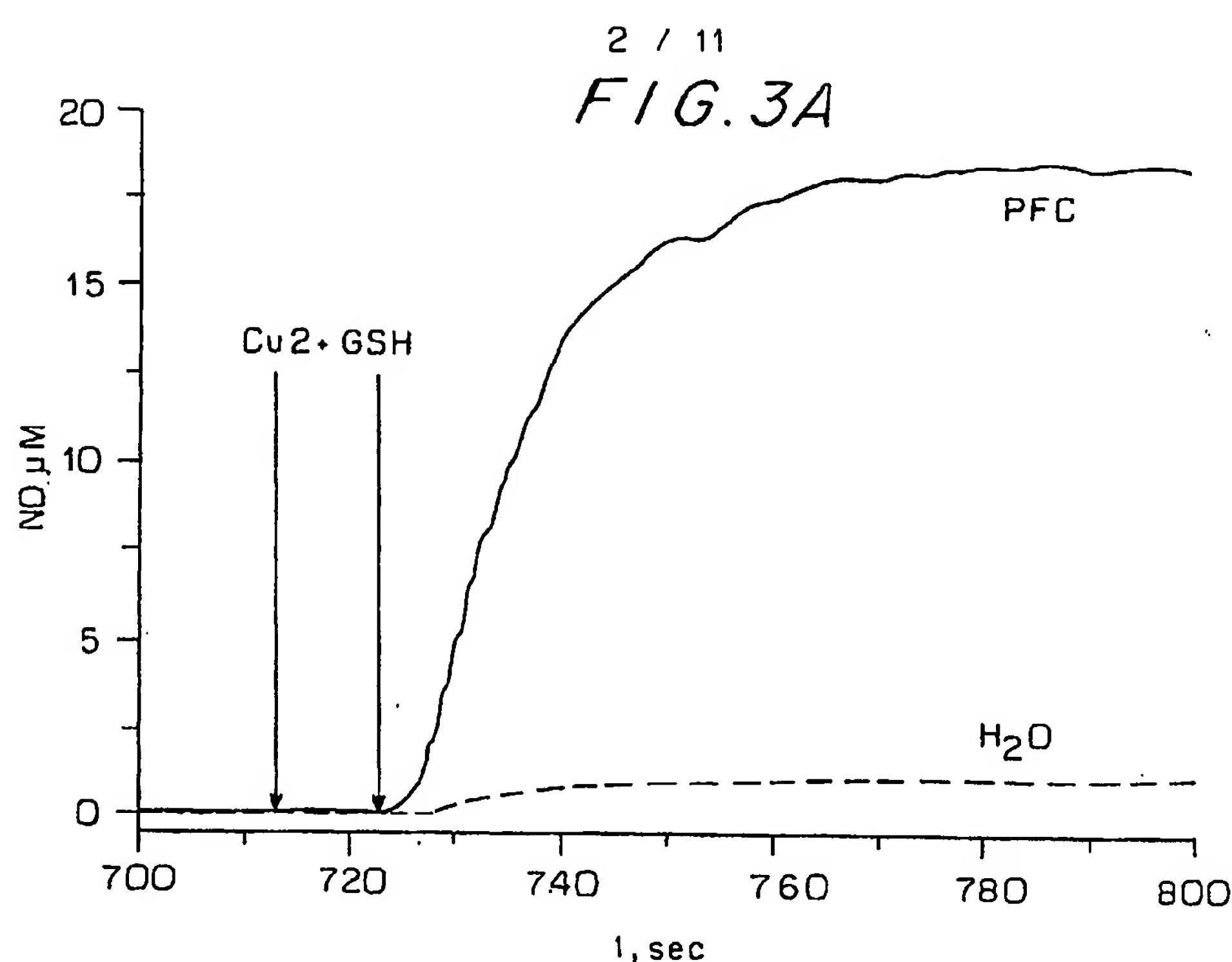


FIG. 2





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FIG. 4A

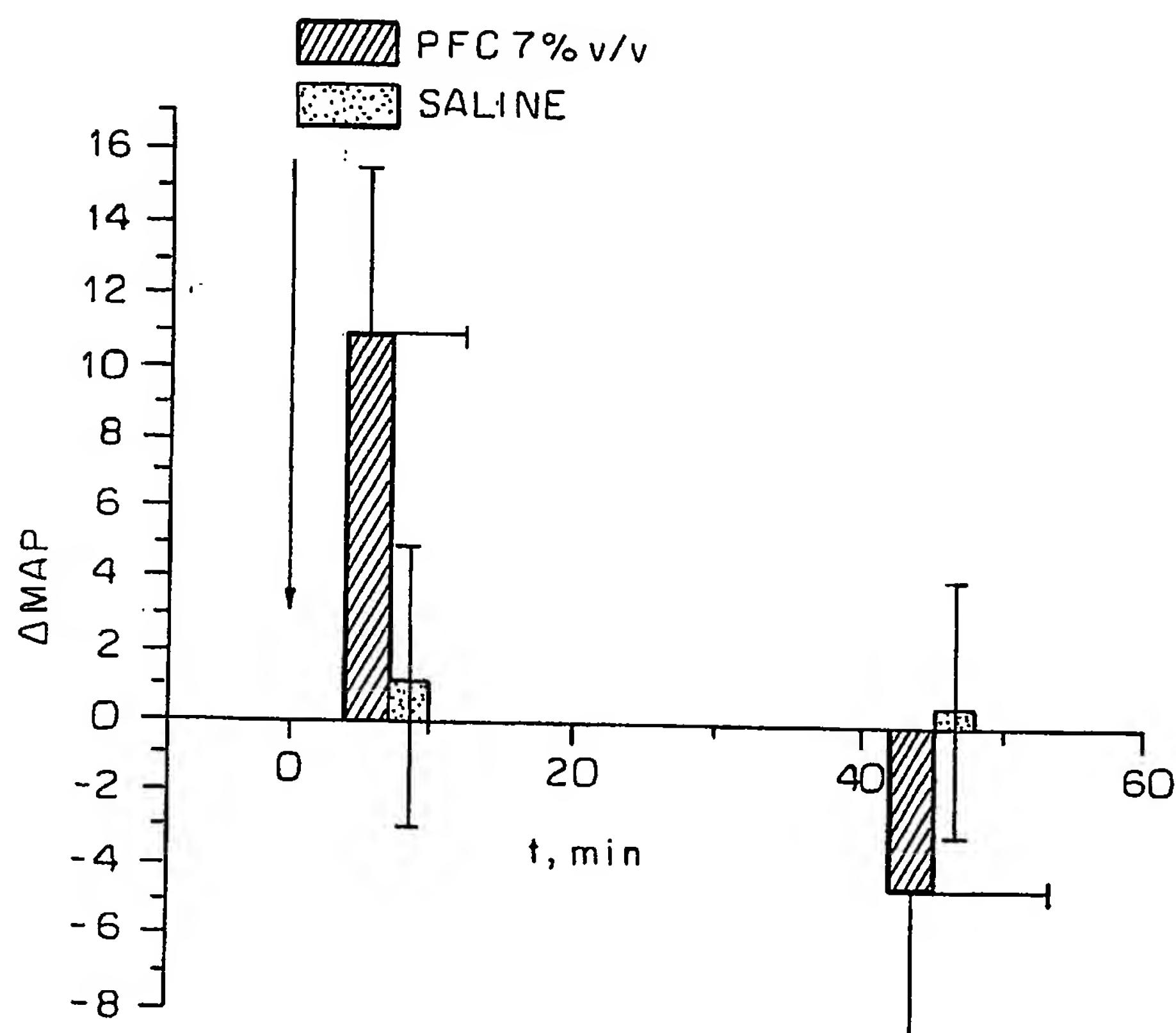


FIG. 4B(1)

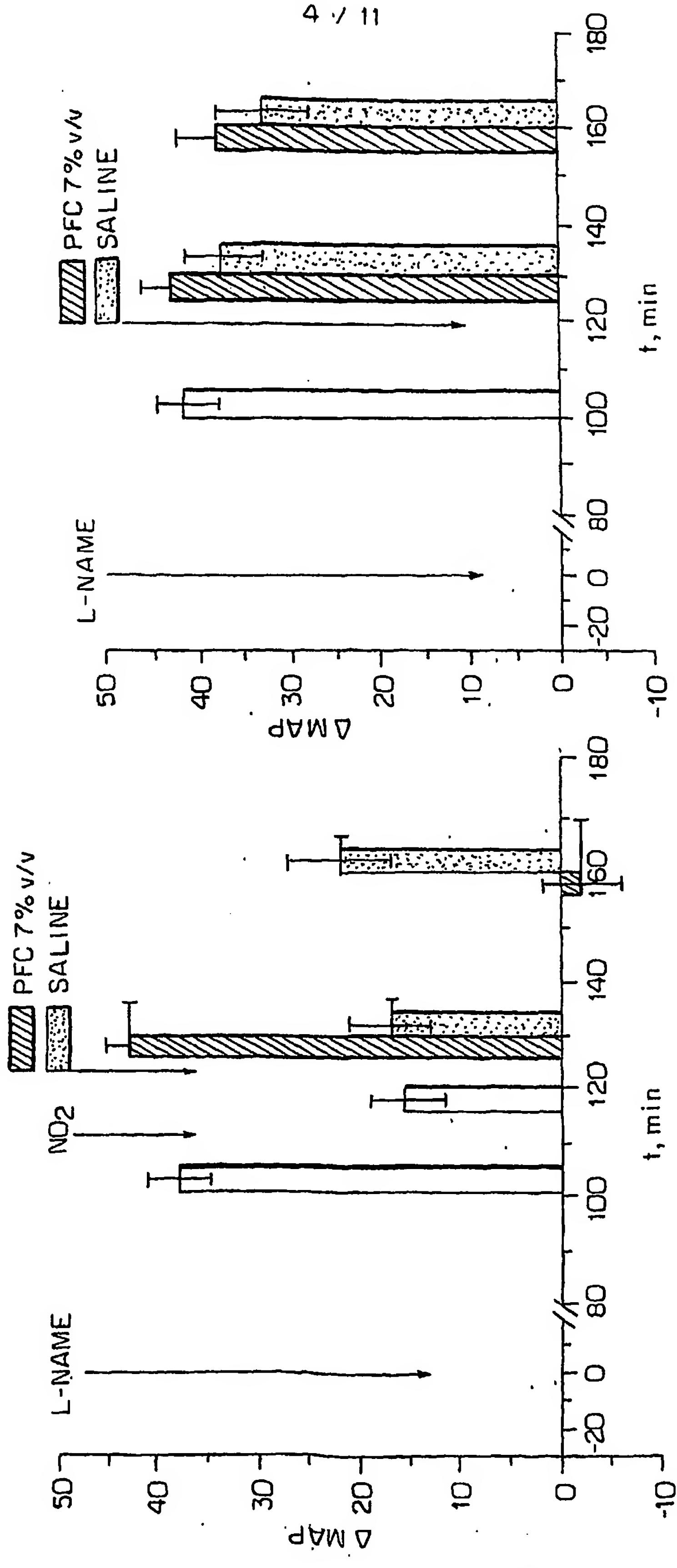
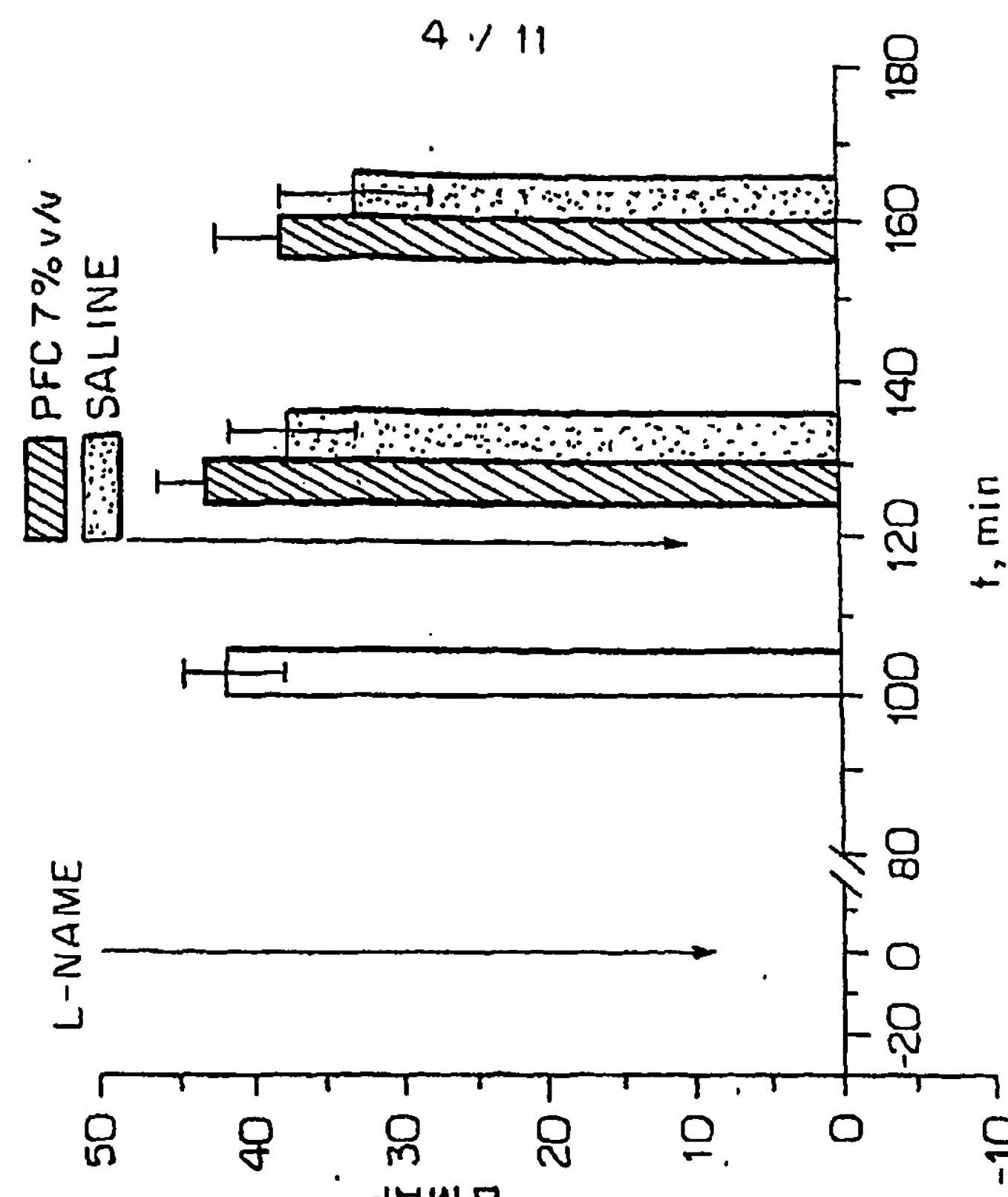


FIG. 4B(2)



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FIG. 5

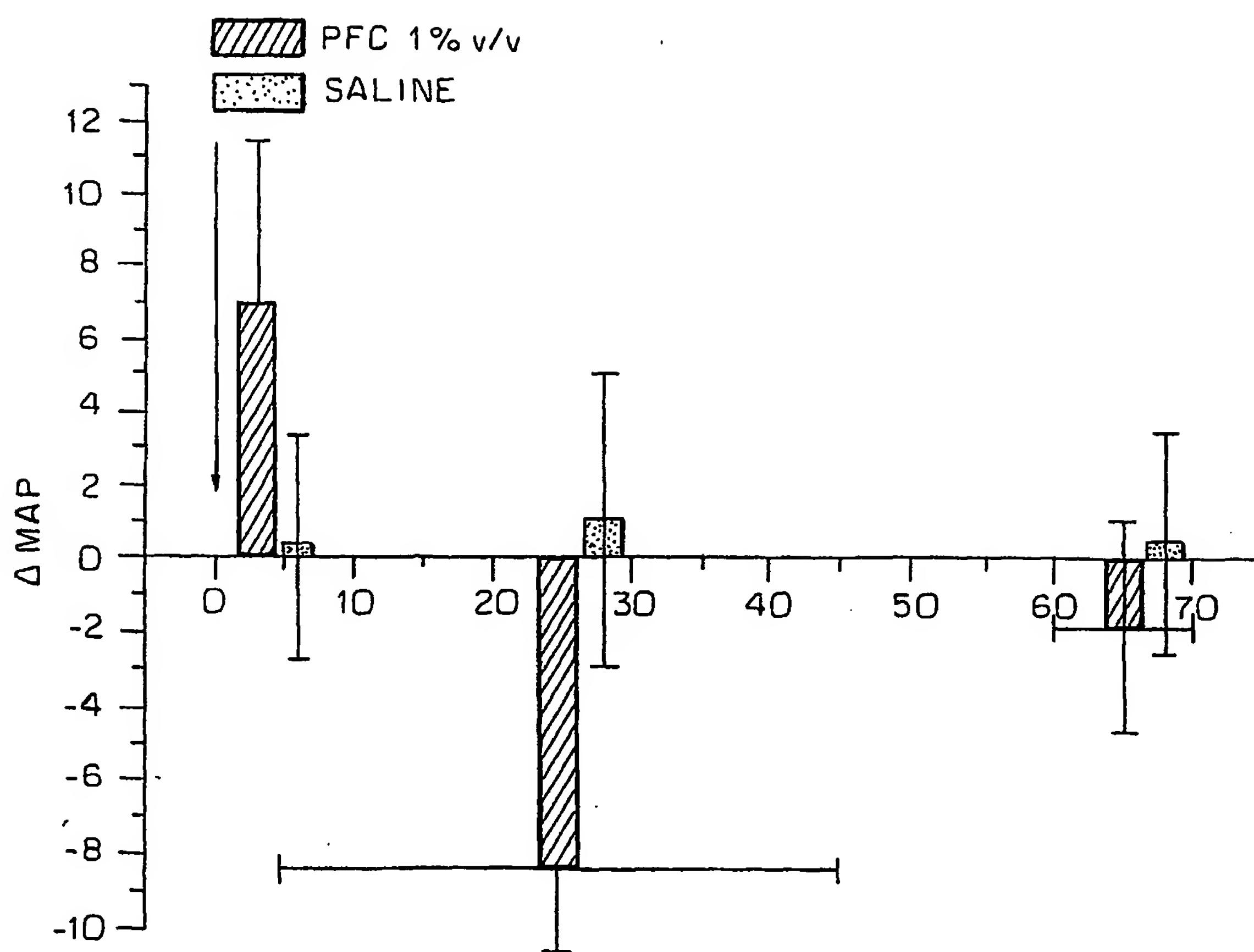
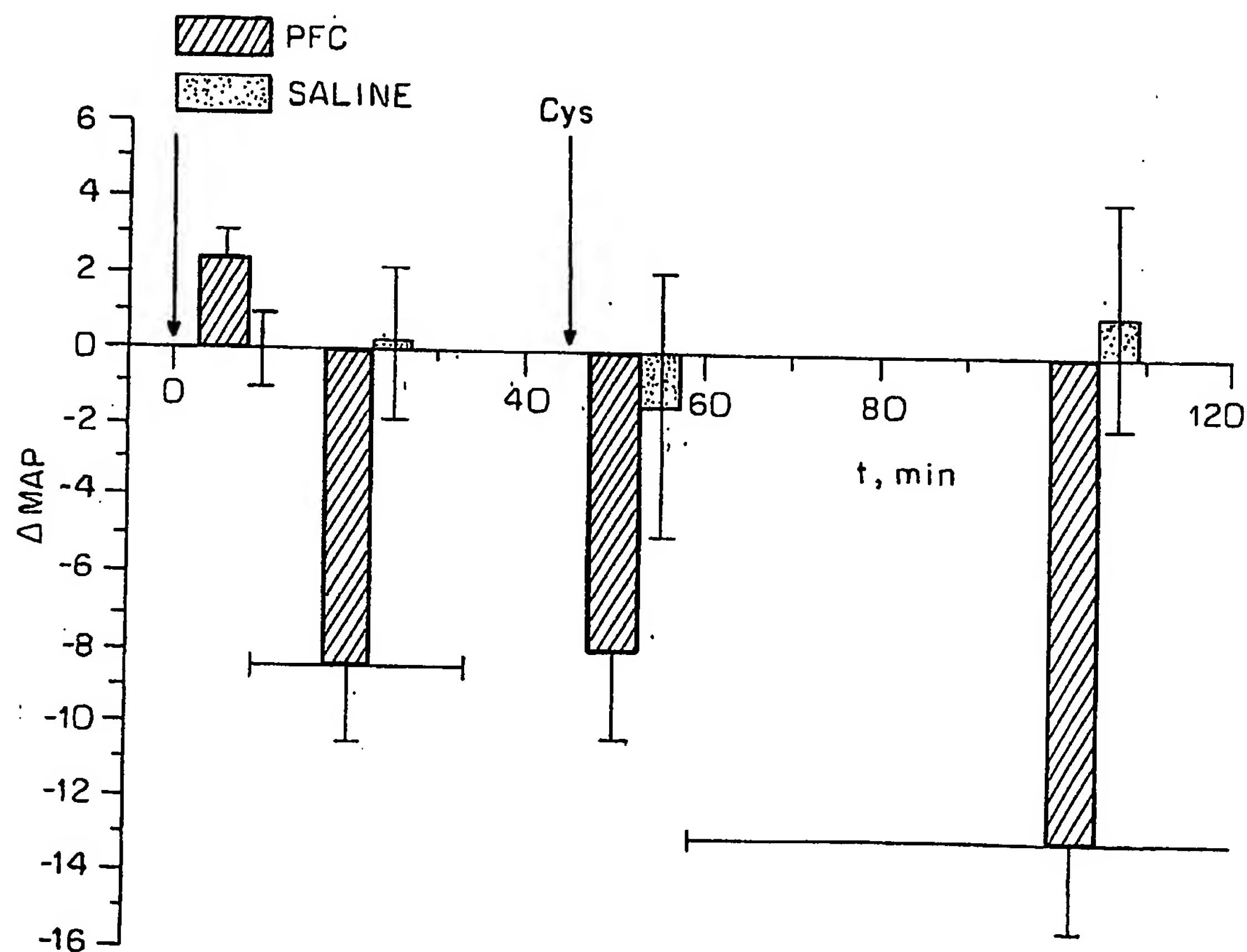
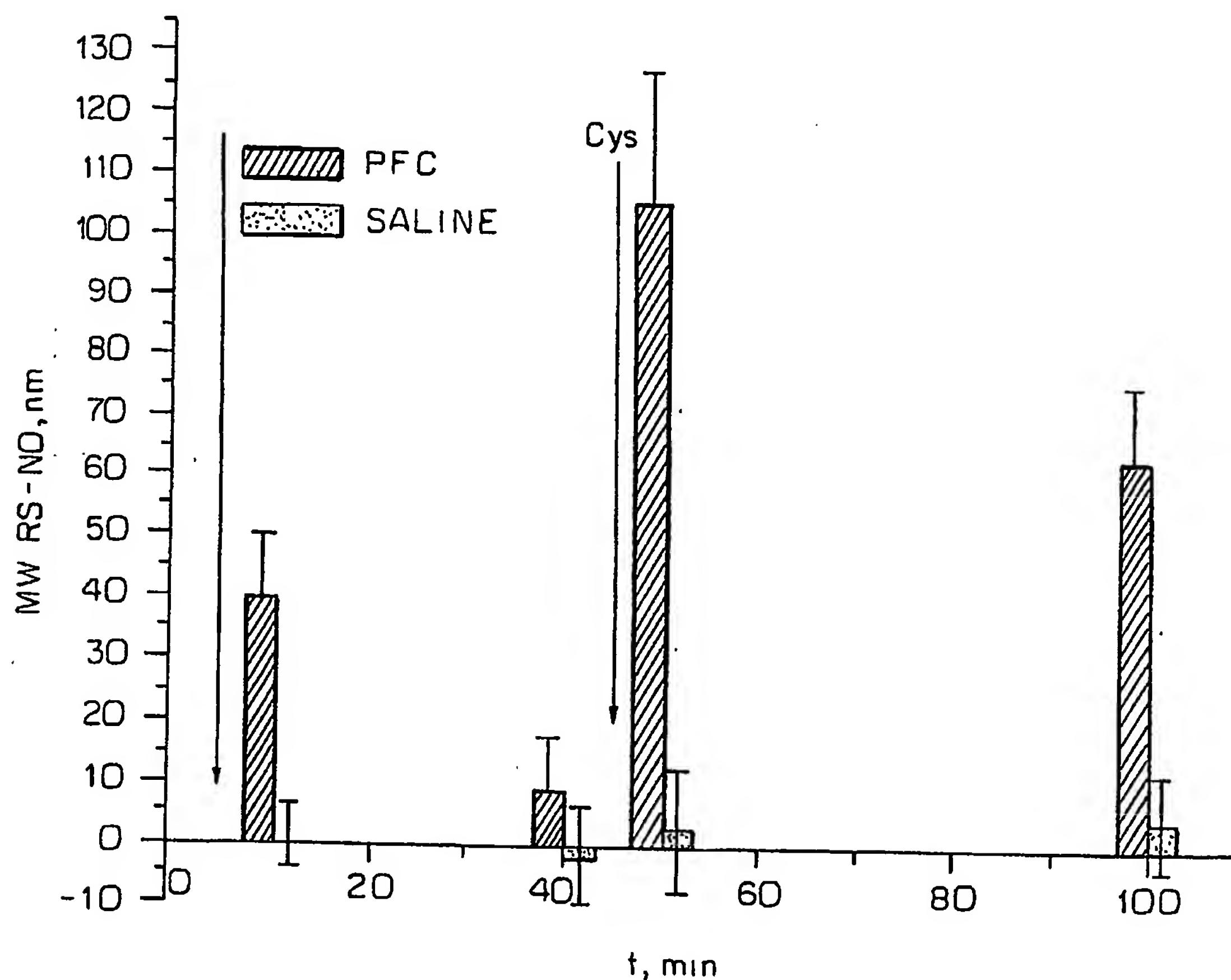


FIG. 6A



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FIG. 6B



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FIG. 7A

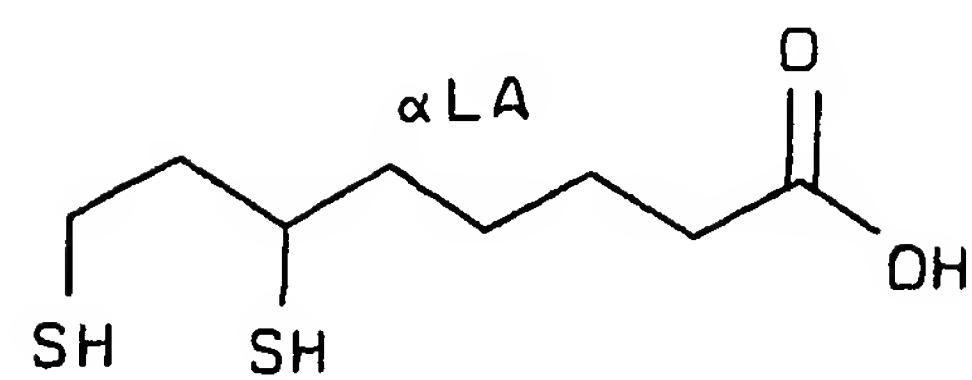
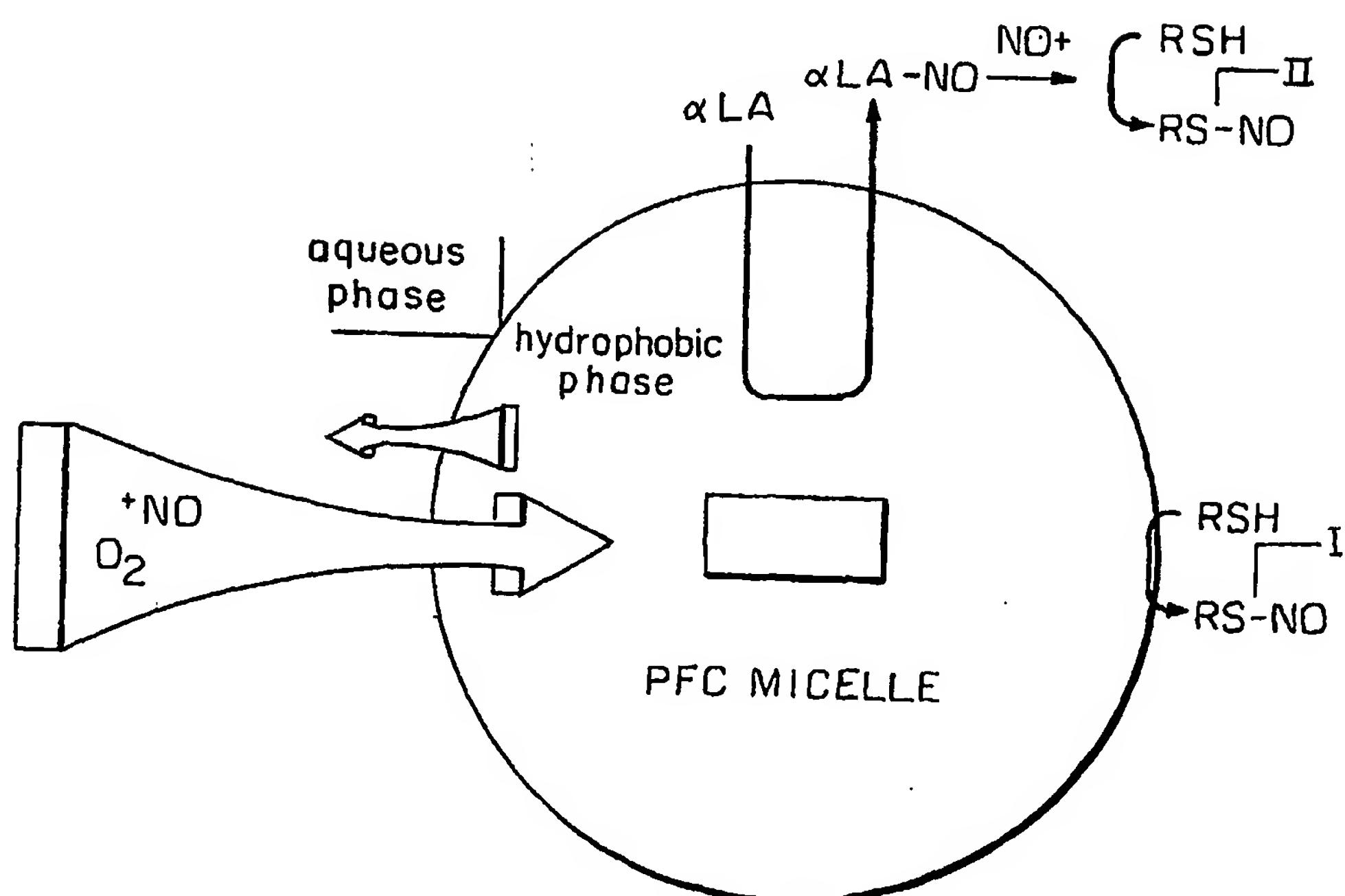


FIG. 7B



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FIG. 8A

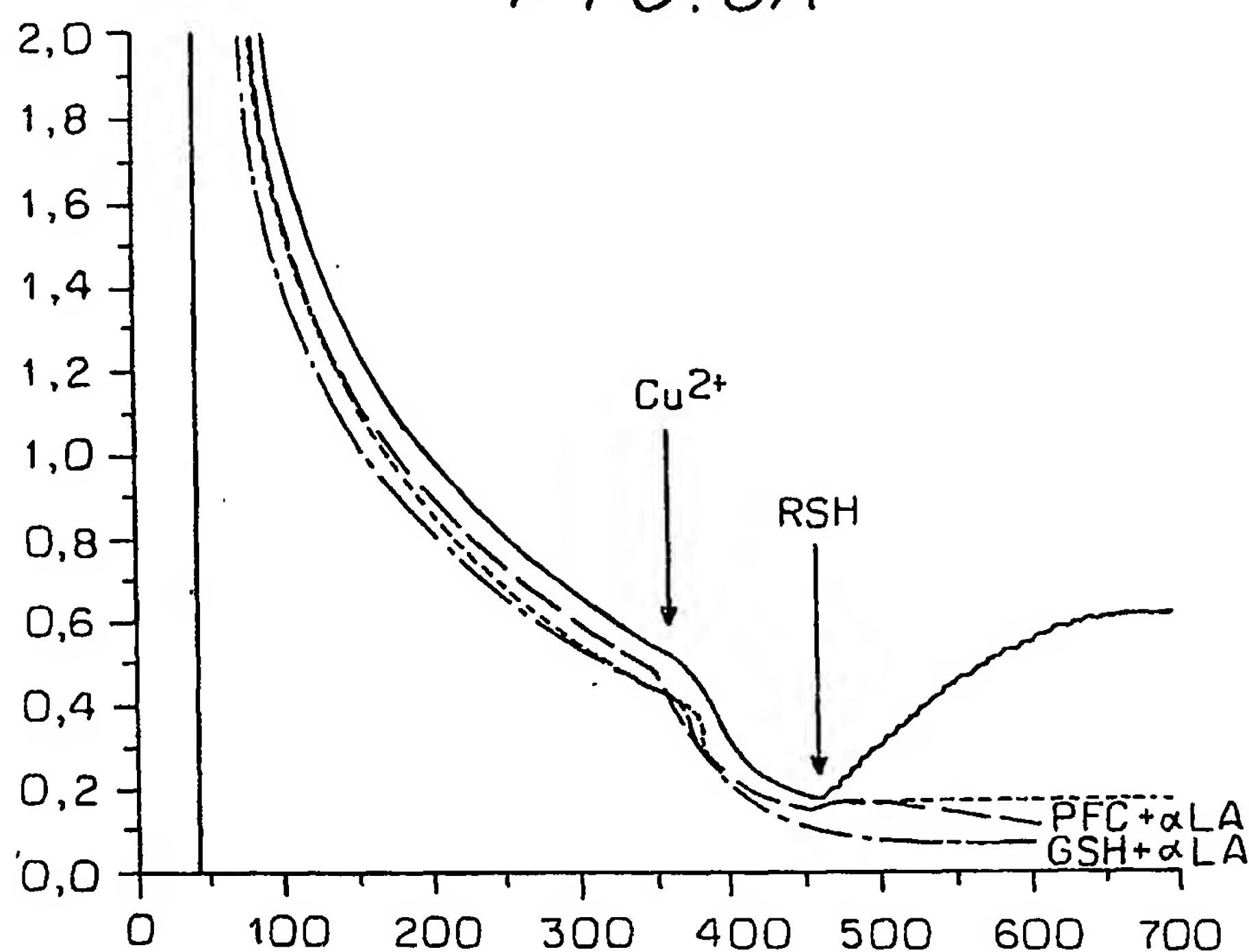
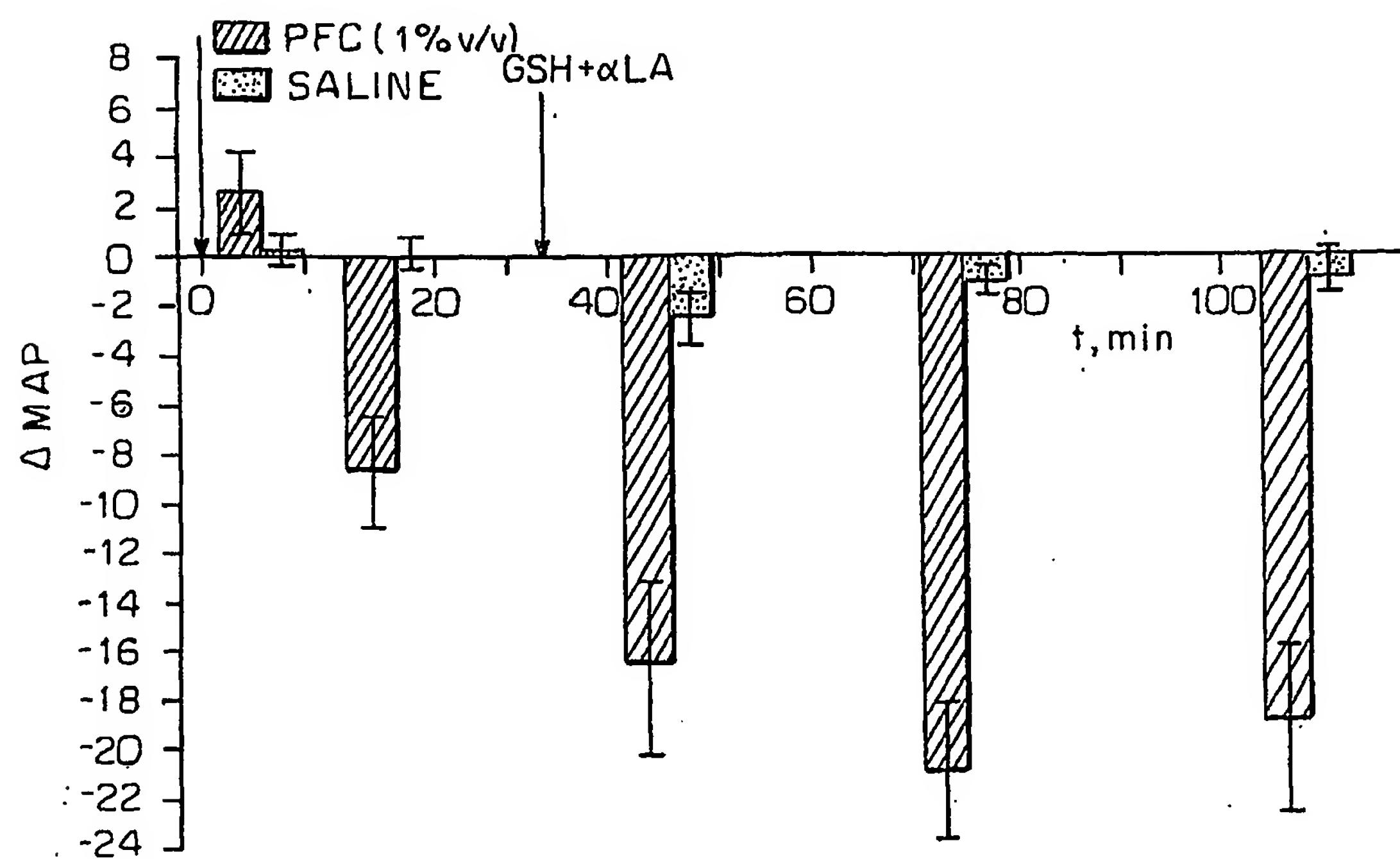


FIG. 8B

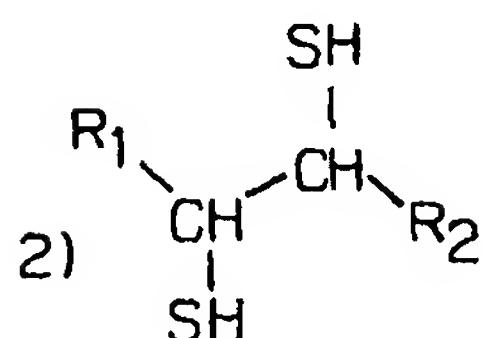


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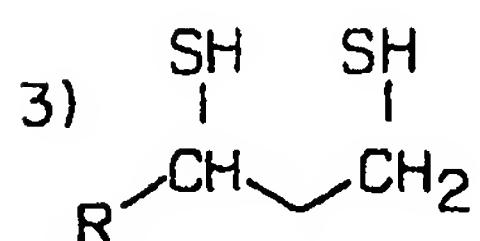
FIG. 9

Thiols

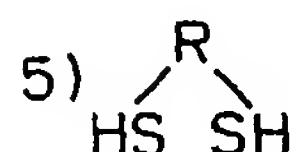
1) R-SH

(R-aromatic, alkyl, peptidil etc)
cystein, homocystein, glutathione

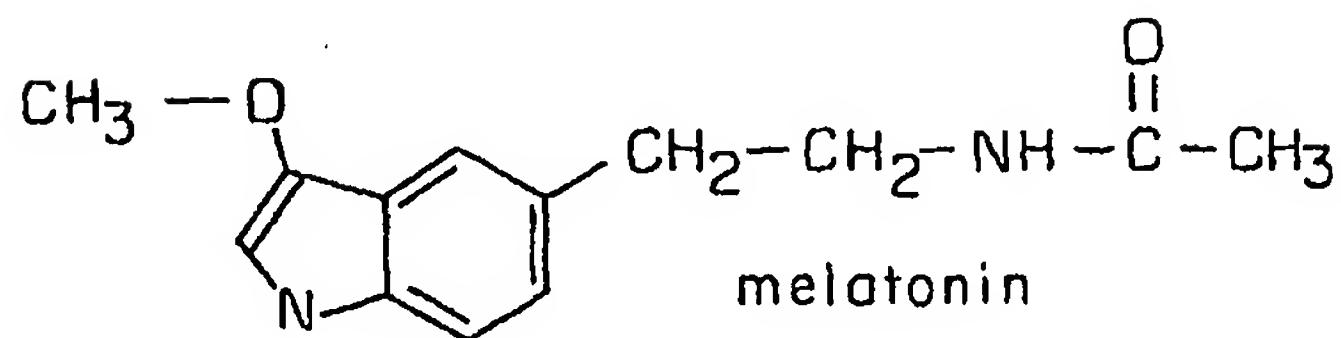
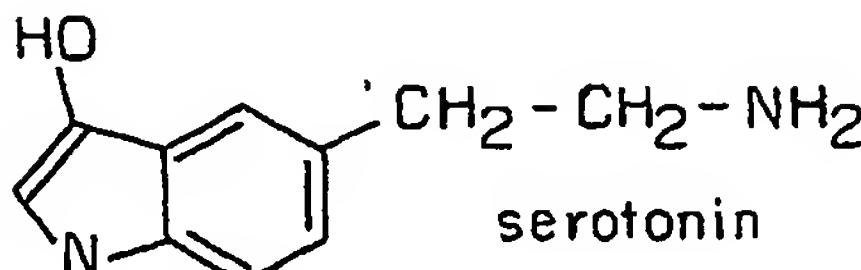
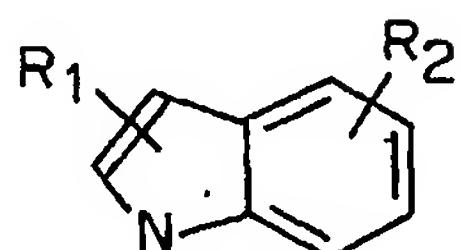
1,2 dithiols

1,3-dithiols (α -lipoic acid)

4) Three thiols ?



Tryptophane like



Piridine, heterocyclic like

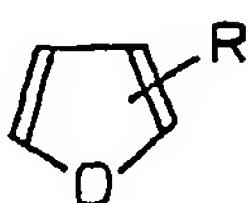
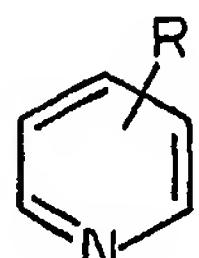
Antioxidants
 α -tocopherol

FIG. 10A

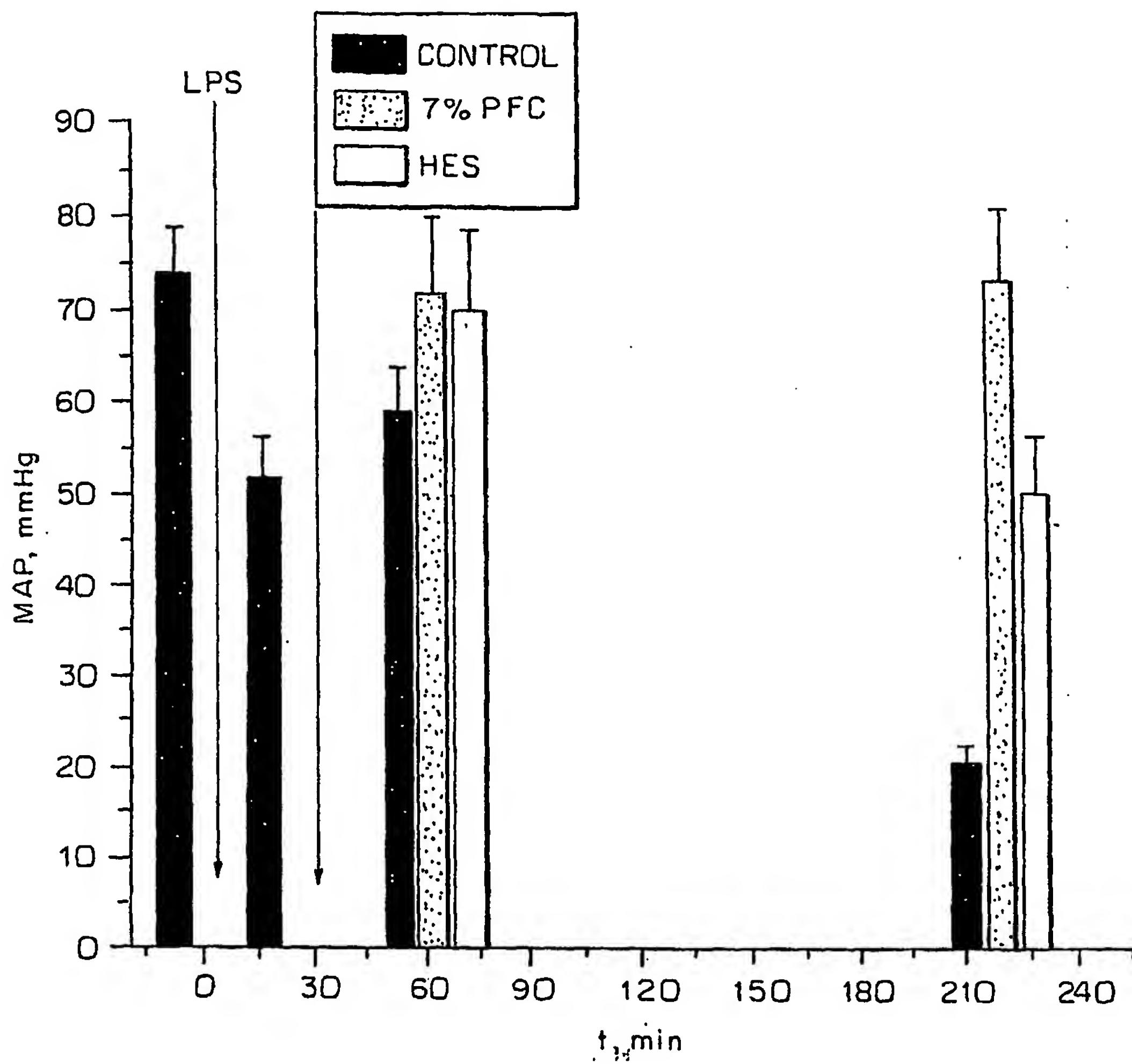
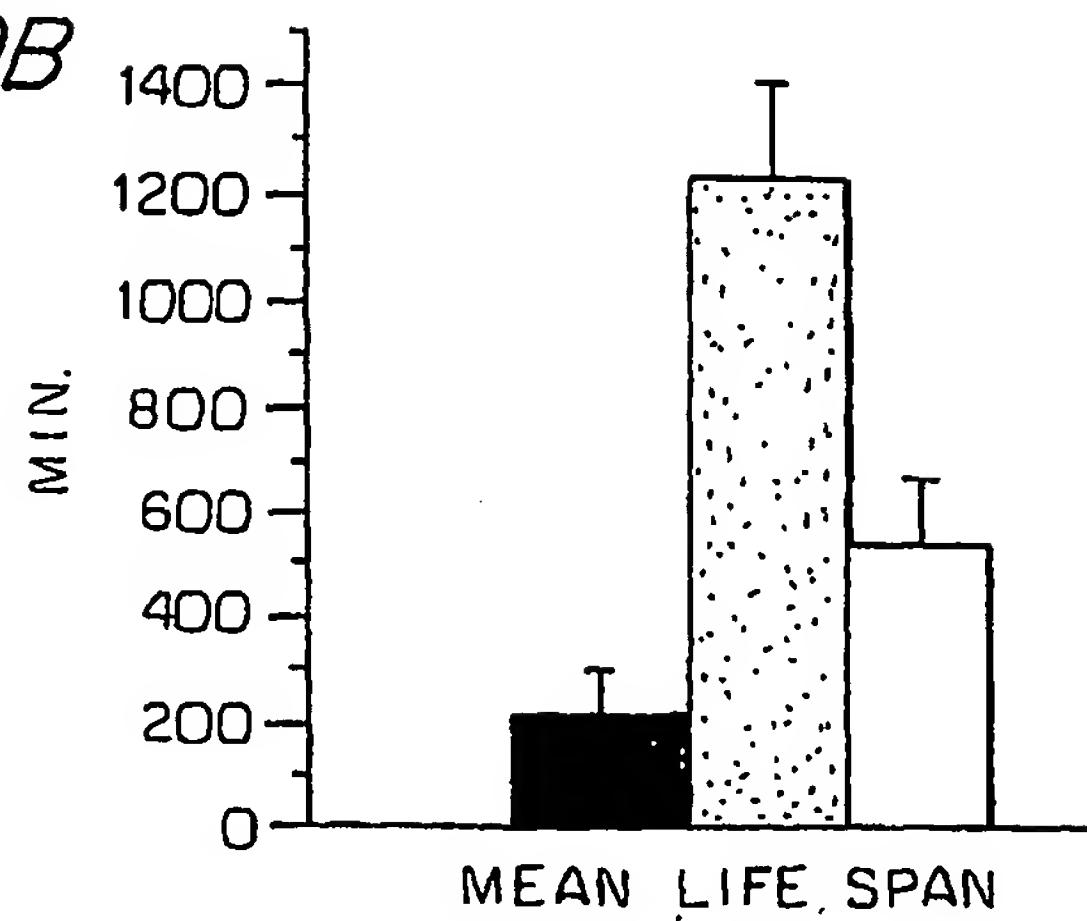


FIG. 10B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/29067

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 49/00

US CL : 424/9.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.11, 1.65, 9.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
registry, biosis, medline, uspatfull, embase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,375,931 B2 (OSTENSEN et al) 23 April 2002 (23.04.2002), see entire document, especially, abstract, column 34-49.	1-27

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
20 November 2003 (20.11.2003)

Date of mailing of the international search report
15 DEC 2003

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